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ERRATA

On page 73, Vol. 78, No. 1, May, 1943, line 2, the word "is" should be inserted after the word "dose."

On p. 77, Vol. 78, No. 1, May, 1943, line 10, the word "statistics" should be changed to "statistic s." The letter s is in this case a statistical symbol and should be italicized.

EFFECT ON THE ELECTRICAL ACTIVITY OF THE CORTEX OF CERTAIN DEPRESSANT AND STIMULANT DRUGS—BARBITURATES, MORPHINE, CAFFEINE, BENZEDRINE AND ADRENALIN[1]

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In 1890, Fleischl von Marxow (1) reported that the electrical activity of the cortex can be modified by chloroform. No great interest was aroused, however, in this or any other aspect of the electrical activity of the cortex until Hans Berger published his classical studies on the human electroencephalogram. The first of these appeared in 1929 (2). A few years later, many other investigators entered this new field, and by 1940, electroencephalography was an established clinical and experimental technique with a bibliography of over 300 titles (3).

The most important facts that have been discovered about the electrical activity of the cortex are the following: The cortex produces fluctuations in electrical potential which can be led off the brain directly or recorded through the unopened skull. These originate in nerve cells of the cortex and not in the blood vessels or glia. Records of cortical activity usually show a strong ten-per-second rhythm with an admixture of many other frequencies. During infancy the dominant activity is slower than ten-per-second; it increases rapidly during the first year, less so thereafter, and attains a nearly adult frequency by the twelfth year. During attention the electrical activity of the cortex becomes faster and during sleep it becomes slower. In various disrurbances of brain function, notably in epilepsy, the electroencephalogram is greatly modified, not only during an attack but usually also between attacks; it can be used, therefore, as an aid to diagnosis. It can be used also to localize cortical lesions, for injured nerve cells tend to beat more slowly (or occasionally more rapidly) than normal. The original reports of these findings can be obtained by reference to lengthy bibliographies published elsewhere (3, 4).

Numerous workers who have studied the effect of drugs on the electrical activity of the cortex (3) agree that most drugs which alter consciousness or produce involuntary movements modify the electroencephalogram. There is no unanimity, however, as to the exact character of the changes that occur, and this is in large part due to the fact that minor but significant alterations are impossible to detect in the unanalyzed record and major changes are often so complex that they defy interpretation or even description The present study was undertaken in the hope that the employment of a precise method of analyzing the electroencephalogram would obviate these difficulties.

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[1] This research was aided by grants from the John and Mary R Markle Foundation and from the Rockefeller Foundation Preparation of records for spectrum analysis was carried out as part of W.P A Project No 22203.

Material and method. Six men were used as subjects. They were selected because they had normal electroencephalograms and no history of brain disease. In order to study the differential action of various drugs, it would be desirable to test each subject on the entire series of drugs under investigation. This could not be done in the present study but whenever possible several drugs were used on the same subject. The drugs and dosages used on each subject are shown in table 1.

The electroencephalogram was recorded as a shadowgram on film, using a monopolar, right occipital lead with the indifferent electrode on the two ear-lobes. The Grass method of frequency analysis (5) was employed. This permits the electroencephalogram to be analyzed into a spectrum, *i.e.*, a plot of the root mean square voltage at each frequency from one to 50 cycles per second. By means of spectrum comparisons it is possible to compare two or more samples of electroencephalogram in minute detail. In order to obtain a spectrum a piece of shadowgram 30 seconds long is cut out at any desired place. The two ends are spliced together so that an endless belt is formed, and this is rotated between a light and a photoelectric cell at a constant speed. The voltage from the photoelectric cell is led through a continuously variable filter circuit, the output of which is recorded on coordinate paper. On one coordinate frequency is registered, and on the other the average amplitude of root mean square voltage at a given frequency.

TABLE 1

SUBJECT	DRUG	DOSE 1	ROUTE	INTERVAL	DOSE 2	ROUTE	INTERVAL	DOSE 3	ROUTE	TOTAL DOSE
		gm			*gm*			*gm*		*gm*
R S	Sodium Phenobarbital	0.32	I V	20 min	0.32	I V	20 min	0.32	I.V.	0.96
J D	Sodium Phenobarbital	0.32	I V	20 min	0.32	I V				0.64
W F	Pentothal	0.09	I V							0.09
J D	Pentothal	0.0625	I V							0.0625
J D	Pentothal	0.09	I V							0.09
W F	Morphine Sulphate	0.016	I V	12 min.	0.016	I M.	50 min	0.011	I M.	0.043
R S	Morphine Sulphate	0.016	Subcut	38 min.	0.016	I V				0.032
A D	Morphine Sulphate	0.016	I M	1 hr	0.016	I M				0.032
S S	Caffeine Sodium Benzoate	0.0487	I V	55 min	0.487	I V.				0.974
J D	Caffeine Sodium Benzoate	0.0487	I V	10 min	0.487	I V.				0.974
A D	Caffeine Sodium Benzoate	0.0487	I M	33 min	0.487	I V				0.974
R S	Caffeine Sodium Benzoate	0.649	I V							0.649
R S	Benzedrine	0.01	I V	10 min	0.01	I V				0.02
M C.	Benzedrine	0.01	I V	10 min	0.01	I V				0.02
R S	Adrenalin	0.0005	I V							0.0005
M C	Adrenalin	0.0005	I V							0.0005

Amplitude and voltage in electroencephalography are usually measured as peak-to-peak amplitude or voltage, which is not the equivalent of root mean square voltage as measured with the Grass frequency analyzer. For example, if the number of waves of a given duration decreases, the alternating current voltage at the corresponding frequency may decrease even though the peak-to-peak voltage of the single waves or bursts of waves may increase. It is true also that all of the alternating current voltage registered at some particular frequency may develop from harmonics of lower frequency waves. Therefore, even though the spectrum shows a relatively high voltage at a given frequency, it might be impossible to detect in the unanalyzed electroencephalogram any waves of that frequency. To avoid confusion, the term *component voltage* will be used to refer to the height of the curve obtained with the Grass analyzer at a given frequency. *Voltage level* will be used to refer to the area of the curve, i e, the sum of the component voltages in a given range *Total voltage level* will be used to mean the sum of all component voltages (total area) between one and 50 per second, the range covered by the present instrument[1]

It might be supposed that differences between spectra could be described adequately in terms of an increase or decrease in component voltage at a specific frequency However, experience indicates that when the component voltage at one frequency decreases the component voltage at some other frequency tends to increase This would not be confusing if at certain fixed frequencies there was a reciprocal relationship in component voltage, but the frequencies which exhibit such a reciprocal relationship vary from case to case and from time to time in the same case These difficulties suggest that changes in component voltage are indeterminate and incomprehensible but this is not so for, as will be shown, if regarded in terms of change in frequency, they appear to have definite characteristics

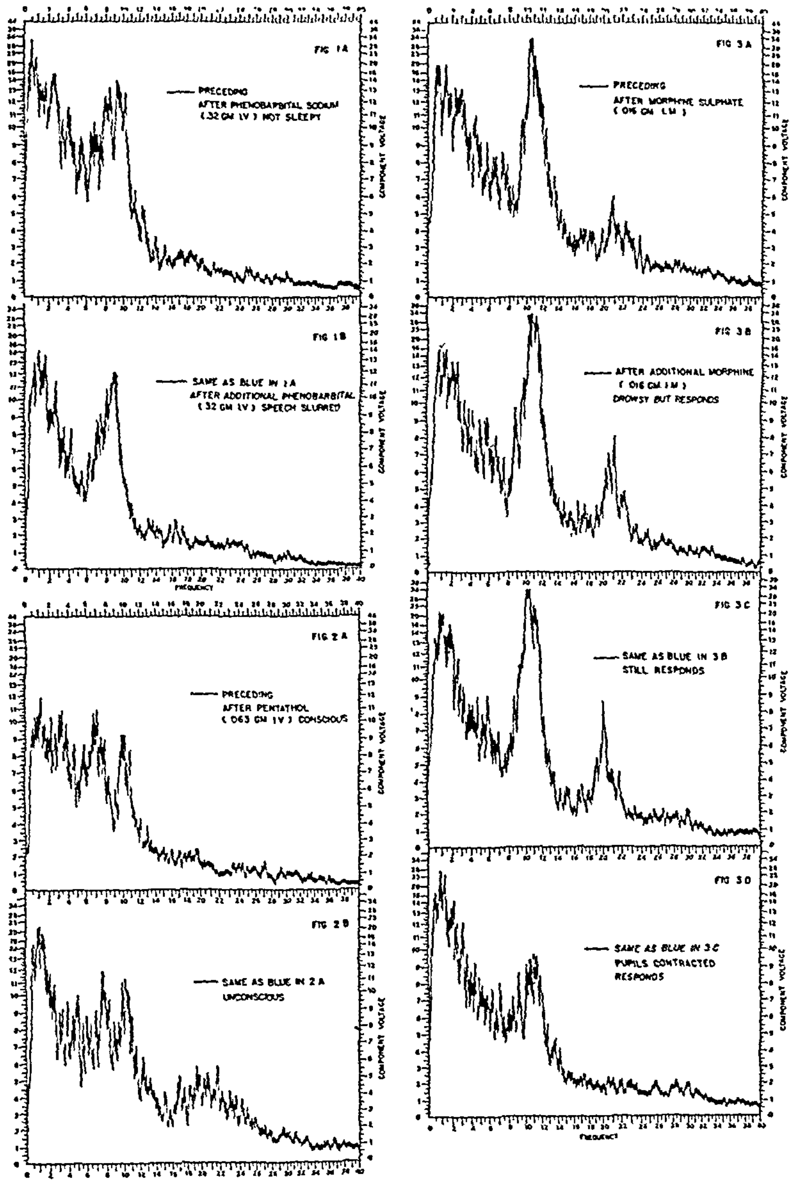
Among the most striking characteristics of cortical spectra are the maxima or peaks of component voltage that appear at certain frequencies In many cases differences between spectra are most easily described by saying that a peak has moved, i e, the summit and slope of the peak are displaced to the fast or the slow side of the spectrum and the entire peak appears to have assumed a new position on the frequency scale When one peak is displaced all peaks tend to be displaced in the same direction, the spectrum is then said to have *shifted* In some cases with complex spectra shifts are difficult to detect and quantify Even if the spectrum has simple and well defined peaks, it may be difficult or impossible to determine the direction and extent of the shift, when it is abruptly and radically modified

The measurement of shifts is carried out as follows A spectrum is placed upon a trans illuminator and a succeeding spectrum is placed under it The attempt is made to obtain maximal superimposition of the two curves by a displacement on the frequency scale The amount of shift is recorded in terms of the degree of displacement necessary to make the second curve coincide as nearly as possible with the first For example, if the spectrum obtained one minute after the intravenous administration of phenobarbital can be made to coincide optimally with the previous spectrum by shifting it one half cycle to the slow side, then the spectrum is said to have shifted one half cycle to the slow side In order to reduce errors due to subjective factors, measurements were made by three independent observers without knowledge of the conditions under which the spectra were obtained A shift was said to have occurred only when all three observers agreed on its direction The magnitude of the shift was taken as the average of the values reported by the three observers

RESULTS *A Depressants Sodium phenobarbital* caused at first a slight decline in the total voltage level (fig 1) and a shift of the highest peak in the 7-to-12 per second range to the slow side In a subject who had at the outset no peak in the 7-to 12 per second range, there was evidence of an increase in voltage level at the slow end and a decrease at the fast end of this range As the effect of the drug progressed, or with increased doses, the 7 to 12 per second peak shifted further and further to the slow side of the spectrum and the total voltage level increased This increase was proportionately greatest in the 14 to 30 per-second range and there was an accompanying shift of the highest peak in this

---

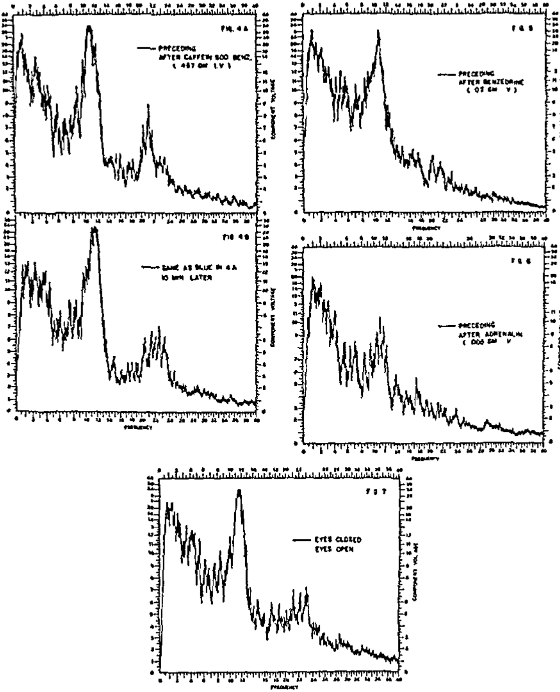
[1] In describing a new phenomenon it is sometimes necessary to devise new terms or to employ old terms in a new sense Either course is hazardous new terms are likely to sound like jargon and old ones have specific meanings that are difficult to modify Much help has been given by Mr Albert M Grass of the Harvard Medical School and Professor E A Guillemin of the Massachusetts Institute of Technology in selecting the present terminology It is hoped that the terms which have been chosen will be more or less self explanatory and will not be confused with expressions that have acquired rigorous scientific meaning



FIGS. 1 TO 7. CHANGES IN SPECTRA FROM THE RIGHT OCCIPITAL AREA PRODUCED BY VARIOUS DRUGS

Frequency is shown in cycles per second, component voltage in arbitrary units. In each figure, the black curve precedes the blue. The part of each curve between 0 and ½ per second is not accurate but is included because it provides some useful information. The part of the curve between 40 and 50 cycles per second adds nothing significant to the data; it is omitted here in order to save space.

region to the slow side. In one of the cases the peak which initially centered on 19 per second moved until it centered on 14 per second. In the case in which there was no peak in this region the voltage level at the slow end of the 14 to-30 per second band increased and at the fast end decreased.



FIGS. 4 TO 7

Pentothal produced the same general changes as sodium phenobarbital except that in the initial stages there was a great increase in voltage level in the 14 to 30 per second range (fig. 2). With further progress in the effect of the drug, or with increased dosage, the broad peak centering on approximately 20 per second moved to the slow side so that it centered at approximately 18 per second. Con currently, the dominant peak in the 7-to-12-per-second range also shifted to the slow side and a new high peak developed in the 6-per-second region. At a later stage, or with still higher doses, a peak appeared in the 13-per-second position and moved to 12 per second as the component voltage at 18 per second declined. Accompanying these changes there was a continuing increase in the voltage level at frequencies below 6 per second. Small doses produced a moderate increase in the total voltage level and large doses produced a great increase.

Morphine sulphate caused changes in frequency which resemble those that occurred with phenobarbital and pentothal. At first there was an increase in the voltage level in the 20-per-second region. This gave rise to a peak which diminished in height as it moved into a slower and slower position (fig. 3). These initial changes in the frequencies above 12 per second were unaccompanied by any change in the lower frequencies. Later, however, as the peak in the 20-per-second region disappeared, a shift to the slow side occurred in the 10-per-second region with an accompanying reduction in the total voltage level.

B. Stimulants. Caffeine sodium benzoate produced a shift to the fast side (fig. 4) with a reduction in the total voltage level. In one case a peak which centered at 10.5 shifted to 11.5, in another a peak centering at 11 per second shifted to 12 per second. In both cases there was some increase in the voltage level in the 14-to-30-per-second range. Consideration of peak positions, however, indicates that this increase was associated with a shift to the fast side. In the case illustrated in fig. 4 a peak which at the start centered on 20¾ per second shifted to the fast side until it centered on 22 per second.

Benzedrine produced a shift to the fast side of the spectrum with a slight increase in the total voltage level (fig. 5). An increase in the voltage level in the 1-to-7-per-second range was interpreted as resulting from a shift to the fast side of peaks which were "off scale", *i.e.*, below 1 per second. The major peak in the 7-to-12-per-second range shifted ½ to 1 cycle to the fast side (in one case from 10½ to 11, in the other from 9 to 10 per second). In both cases there was an increase in the voltage level in the 14-to-30-per-second range with a movement of peaks in this range to the fast side.

Adrenalin produced changes in frequency similar to those produced by caffeine and benzedrine but more extreme. In one case the center of the dominant peak in the 7-to-12-per-second range was shifted from 10.5 to 12 per second (fig. 6). There was a general increase in voltage level but this was most evident in the 14-to-30-per-second range. The peak which at the start centered at 18 per second shifted its center to 20 per second.

In order to contrast the changes produced by stimulants with changes produced by attention, the effect of opening the eyes in the light is shown in fig. 7. The changes in frequency and voltage level occurring in figs. 1 to 7 are summarized in fig. 8, and for purposes of comparison, an example is shown of the changes that occurred in one subject during normal sleep.

Discussion. Although the cortical frequency spectrum is a simplified representation of the electroencephalogram, it still shows the electrical activity of the cortex as a complex phenomenon. Many things can be seen, however, in a series of spectra that are invisible or only dimly apparent in the unanalyzed electroencephalogram . By choosing for study cases with clearly-defined peaks in certain regions, it is possible to observe and demonstrate shifts in frequency. In some cases with complex spectra the changes are not sufficiently clear to be comprehended by an inexperienced observer but any two experienced observers would agree on the character and direction of the change In some cases where there are no peaks or where the spectrum is abruptly and radically modified, it is impossible even for the most experienced observer to decide what has happened.

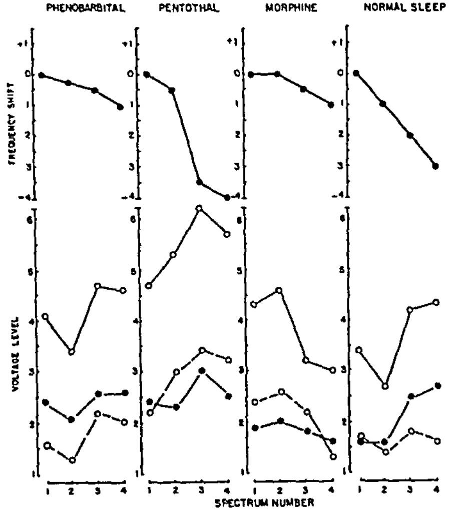


Fig 8 Constant Shift in Frequency and Variable Changes in Voltage Level Produced by Sedatives

[illegible]

the increasing effect of the drug or, in the case of sleep,
I on each curve is shown the value for the preceding c
frequency shift, heavy broken line, total voltage level, light broken line, voltage level between 1 and 10 per second, dotted line, frequency level between 10½ and 50 per second

All three of the depressants that were studied produced a shift in frequency to the slow side. The increase in voltage level in the 20-per-second range was believed to be similar to the change that occurs in a light stage of normal sleep when 14-per-second activity becomes prominent, and it was interpreted as a piling up of voltage in this intermediate range as a result of slowing in still faster frequencies.

Pentothal was the only drug in the series that produced unconsciousness. The associated shift in frequency of four cycles per second to the slow side is comparable to that which occurs in deep normal sleep. A shift of one cycle per second, such as was produced by phenobarbital and morphine, can occur "spontaneously" in normal subjects without clinical signs of altered central nervous function (6, 7).

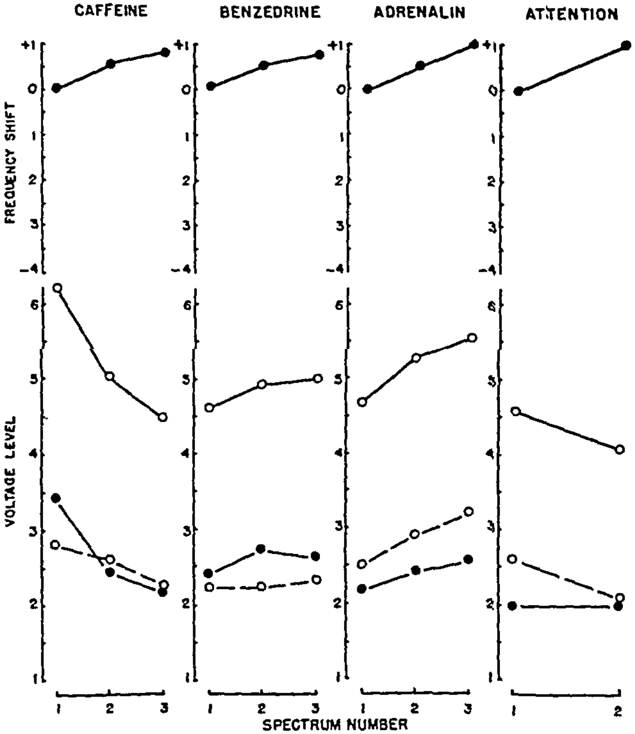


FIG. 9. CONSTANT SHIFT IN FREQUENCY AND VARIABLE SHIFT IN VOLTAGE LEVEL PRODUCED BY STIMULANTS

Ordinates and abscissas, and designation of curves as in fig. 8. In the set of curves marked *attention*, the first values are those for eyes closed and the second those for eyes open while reading.

All three stimulants produced a shift to the fast side; adrenalin was most effective, benzedrine less so, and caffeine least, but none were more effective than attention. The shift to the fast side that occurs with attention is possibly the maximum that can occur without gross disturbance of central nervous function. Observations on epileptics indicate that a shift of more than one cycle per second to the fast side is likely to be associated with convulsions (8).

It might be assumed that these depressants and stimulants effect the cortex indirectly, the former by causing drowsiness and the latter by increasing attention. The neurophysiology of sleep and attention are so little understood, however, that such an assumption does not lead to a simple explanation.

A relationship was evident between the degree of shift and the concentration of the drug, but no general relationship could be made out between changes in voltage level and drug concentration. It might be assumed that the lack of relationship between voltage level and drug concentration was the result of the selection of arbitrary frequency ranges and that if the voltage level in wider or perhaps narrower frequency bands had been considered, a direct and simple relationship might have been demonstrated. Much experimentation with the data, however, has failed to reveal any specific frequency bands which give better relationship than those shown in fig. 8.

Shifts in frequency to the slow side are usually associated with an increase in total voltage level, and shifts toward the fast with a decrease, but there is no fixed relationship between frequency and voltage level. This is in part explained by the fact that at certain "resonant" frequencies the component voltage is maximal and a frequency shift in either direction will be associated with a decrease in voltage. Despite its apparent inconsistency, voltage level is an important variable which reveals significant differences between drugs of the same class. These differences center around differences in total voltage level and also around differences in component voltage in specific frequency bands, *i.e*, one drug has a major effect on one range of frequencies and another on another. Furthermore, with various drugs there are differences between the order in which a given sequence of changes occur. It is possible that if a larger number of drugs were studied some of these differences could be correlated with differences in the chemical structure of the drugs or with differences in the effect of the drugs on intellectual, sensory and motor functions. This much can be said from the present data—the substance that caused the most extreme shift to the slow side (pentothal), caused the most definite impairment of consciousness. The substance that produced the most extreme shift to the fast side (adrenalin) produced the most evident symptoms of excitement. The depressant that produced the most evident increase in activity in the 14-to-30-per-second range (pentothal) produced the most involuntary muscle movement.

SUMMARY AND CONCLUSIONS

The effects on the electrical activity of the cortex of phenobarbital, pentothal, morphine, caffeine, benzedrine and adrenalin have been investigated. Six subjects without brain disease were used. In order to study the effect of different drugs on the same individual all subjects were tested with several drugs. The Grass method of frequency analysis was employed to transform into a spectrum the electroencephalogram from the right occipital area, recorded with an indifferent electrode on the lobes of both ears Series of spectra were compared in order to detect the changes that resulted from the administration of each drug. As a result of these comparisons, the following observations were made:

1) Phenobarbital, pentothal, and morphine caused a shift in frequency to the slow side comparable to that occurring in sleep

2) Caffeine, benzedrine, and adrenalin caused a shift in frequency to the fast side comparable to that occurring with attention.

3) Pentothal caused a great increase in the voltage level in the 14-to-30-per-second range during the initial stages of its action; morphine produced less of an increase, and phenobarbital, least.

4) Of the drugs studied, the one (pentothal) which produced the greatest clinical evidence of depression, caused the greatest shift to the slow side, and the one (adrenalin) which produced the greatest evidence of stimulation, caused the greatest shift to the fast side.

5) The direction of the change in frequency was constant for a given class of drugs but the direction of the change in voltage level varied with dosage and differed for drugs of the same class.

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A COMPARISON OF THE ACUTE AND CHRONIC TOXICITY OF CARBON DIOXIDE WITH ESPECIAL REFERENCE TO ITS NARCOTIC ACTION[1]

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It is well established that CO_2 exerts a narcotic action on certain functions of the central nervous system when this gas is inhaled in moderately high concentrations (1, 2, 3, 4) This effect may be due to the increase in hydrogen ion concentration resulting from the uncompensated respiratory acidosis and the condition is somewhat analogous to the cerebral depression and coma which commonly occur coincidentally with an accumulation of acid metabolites The available evidence indicates that this narcotic action is associated with, and probably results from, a reduction in cellular oxidations, a concept which would receive theoretical support from the known inhibition of many oxidative enzymes with increase in hydrogen ion concentration Since the literature regarding the narcotic action of CO_2 is scanty and the conclusions vague, the problem is deemed worthy of further investigation

METHODS In most experiments the albino rat served as the experimental animal although a few rabbits and dogs were used The acute and chronic toxicity was determined by placing animals in a closed circuit system of 1100 liters capacity consisting of a 600 liter animal chamber, a 100 liter spirometer and two tanks of 200 liters each The atmosphere in this system was circulated by a small blower, the concentration of O_2 was maintained at approximately 21% In chronic experiments the concentrations of CO_2 and O_2 varied from the stated levels as much as 2 to 3% during any 24 hour period

The oxygen consumption of the rat was determined in an airtight 6 liter glass chamber suspended in a water bath maintained at 28°C The brass cover contained, in addition to openings for a thermometer, a mercury manometer and a sampling outlet two large stop cocks through which gas mixtures from the large system could be circulated continuously Access to a small wire animal cage suspended from the cover was obtained through a 3 inch opening with an airtight rubber seal Gas entering the small chamber from the large system was saturated with water vapor at 28°C as it passed through a large brass coil and shower type humidifier, both of which were suspended in the bath Circulation within this chamber when it was isolated from the large circuit, was obtained with a small electric fan placed entirely within the chamber In making the determination the rat, fasted 18 hours was placed in the chamber one hour before the run The gas mixture containing the desired amount of CO_2 and O_2 was then circulated through the small chamber until a uniform atmosphere was attained (15 minutes) a gas sample was taken, and the ports closed One half hour later another gas sample was taken, the ports opened and the gas from the large system allowed to circulate for at least 15 minutes before the process was repeated

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Both the gas samples were analyzed in the Van Slyke manometric apparatus, and calculations were made of the quantities (S.T.P.) of CO_2 produced and O_2 consumed.

The effect of exposure to 11% CO_2 on the acid-base balance of the blood was determined on arterial blood samples collected from rats anesthetized with 45 mgm. per kgm. of sodium pentobarbital. One hour after injection of the drug, and after exposure to the desired atmosphere for the stated intervals, the blood samples were obtained by aortic puncture after laparotomy. The animals remained in the same atmosphere during the sampling procedure. The blood samples were analyzed by the micro method of Shock and Hastings (5) and the pH corrected for rats' blood at 38°C. Control determinations in room air were made by the same general procedures.

The rate of disappearance of bromsulfalein from the blood of rabbits was used as a criterion of the effect on liver function of acute and chronic exposure to CO_2. The method used was the modification reported by Tatum, Nelson and Kozelka (6). In the acute experiments the rabbits were exposed to 10–15% CO_2 for 2 hours before the test, while in the chronic experiments the rabbits had been in 15% CO_2 for one week. The animals were maintained in the CO_2 atmosphere during the test period.

RESULTS. *A. Toxicity of Carbon Dioxide. 1. Following sudden exposure.* The acute maximal tolerated concentration of CO_2 for our albino rats lay between 10 and 20%. All animals survived 10% CO_2 indefinitely, an occasional one succumbed in 15%, but 80% of rats placed in 20% CO_2 died within 4 days. None survived in 25% CO_2 for more than 36 hours, whereas 50% CO_2 was uniformly lethal within 6 hours. In all cases the oxygen concentration was maintained at 21%.

The rats were narcotized immediately when placed in concentrations of CO_2 above 30 per cent. A detectable grade of depression occurred with concentrations as low as 20% although the animals were not deeply narcotized. Death from carbon dioxide was always associated with evidence of pulmonary injury. The lungs were found to be edematous and filled with a sanguineous exudate when examined at autopsy. In the higher concentrations similar changes also occurred in the exposed mucous membranes. In the rabbit, transient convulsions were produced by concentrations of 25% CO_2 and above, followed by a marked depression.

2. During prolonged exposure. Ten rats were placed in the large animal chamber, supplied with food and water, and the concentration of CO_2 raised immediately to 10% where it was maintained for 30 days. The oxygen concentration was maintained between 19 and 21%. The usual respiratory response to excess CO_2 was the only objective sign observed, since the rats appeared normally active, and young born under these conditions were apparently normal. However, a considerable weight loss occurred, ranging from 14 to 27%. This loss of weight is believed to be due primarily to a reduction in food intake since, as will be discussed later, animals kept at higher concentrations ate sparingly or refused food entirely. Although no studies were made to determine the possible rôle of dehydration, it is perhaps significant that diuresis occurred during acute exposure to CO_2. In view of the good general condition of the animals when they were removed, it is believed that this experiment could have been much prolonged without death of the animals.

Blood studies were made on five of these rats at weekly intervals by Miss

Ethel Thewlis and Dr. O. O. Meyer, of the Department of Medicine, who found a marked reticulocytosis as the only significant change. These results on the rat differ from those obtained on the rabbit and dog by other investigators (7, 8, 9) since we observed no significant change in hemoglobin levels or in the number of leukocytes or erythrocytes.

In another experiment the CO_2 was allowed to accumulate gradually over a 5-day period until a concentration of 20% was reached. The rats were maintained at this level for 6 days and the concentration was then allowed to increase to 25% during the next 3 days. One animal died after 4 days at this level. At this time the concentration was reduced to 20% since the survivors were greatly depressed and a serosanguineous exudate appeared on the exposed mucous membranes. A similar situation occurred when another attempt was made to maintain the concentration at 25%. In this instance, the concentration was reduced to 23% and maintained for 10 days longer—a total of 29 days survival in concentrations of CO_2 varying between 20 and 25% (oxygen, 19 to 21%). The rats ate sparingly at CO_2 concentrations above 10% and food was refused above 23%. During the 34-day experiment, 50% of the original body weight was lost. It is doubtful if this experiment could have been continued much longer with survival of the animals.

When this group of animals was removed to room atmosphere they became very irritable and developed moderate tetany with episodes of mild clonic convulsions. This state lasted for 12 hours but no permanent effects were observed. The animals gained weight rapidly and had recovered completely within a few days. The action of these animals is in direct contrast to that observed in rats removed after 5 hours of acute exposure to 25% CO_2. The latter were much more depressed during exposure and no convulsive manifestations were evident on removal; in fact, the depression produced by a given concentration of CO_2 was always greater upon sudden exposure than during prolonged exposure.

The toxicity of CO_2 is approximately the same for certain other laboratory animals as it is for the rat. Rabbits were depressed very significantly by a concentration of 27% CO_2 built up slowly over a period of 72 hours. A sanguineous exudate appeared on exposed mucous membranes but no convulsions were noted on removal. Dogs were partially narcotized by a concentration of 23% CO_2, even when this level was reached by slow accumulation over a 5-day period. No convulsions occurred when the dogs were removed to room air.

B. Effect of Carbon Dioxide on Oxygen Consumption. 1. Following sudden exposure. The oxygen consumption of rats determined at various intervals after exposure to 11% CO_2 is shown in table 1. Numerous control runs in air and in an atmosphere of 11% CO_2 and 21% O_2 indicate that the method is accurate to ± 3%. The values for the oxygen consumption obtained in these runs checked well with data in the literature obtained by other methods (10), although the results were slightly higher since the rats were kept at 23° to 25°C.

The oxygen consumption diminished immediately after exposure to 11% CO_2 and continued to decrease during the first several hours, reaching a minimum in between two and one-half and five hours. In some animals the oxygen consumption was reduced to 75% of the control value at this time, and with continued exposure a gradual increase in oxygen consumption toward the control level occurred, until at 24 hours the latter was usually regained and in some instances exceeded. In one experiment in which a rat was exposed to a higher level of CO_2 (20%), there was a much greater decrease in the oxygen consumption during the first few hours, and on continued exposure for 48 hours the oxygen consumption did not regain the basal level but remained 15 to 20% below it.

2. During prolonged exposure. The oxygen consumption of rats determined at several concentrations of CO_2 during prolonged exposure is shown in figure 1. The CO_2 content of the atmosphere was allowed to increase at a constant rate of 1.5 to 2% per 24 hours and was maintained at each concentration 24 to 48 hours before the determination of oxygen consumption was made. No change in oxygen consumption was observed until the concentration of CO_2 was greater than 10%. It was reduced to approximately 80% of normal by 15% CO_2 and to 71% of normal by 20% CO_2. During the 19 days of this experiment a 25 to 30% weight loss occurred. Since no significant loss in weight or reduction in oxygen consumption was noted until the concentration of CO_2 exceeded 10%, it is possible that the reduction in oxygen consumption which occurs during exposure to 15 and 20% CO_2 may be related in part to the diminished food intake.

TABLE 1

Oxygen consumption following sudden exposure to CO_2 at 28°C.*

RAT	CONTROL	½ hr.	1½ hr.	2½ hr.	3½ hr.	4½ hr.	15 hrs.	24-72 hrs.	MAXIMUM REDUCTION
									%
1	81	73	68	70		59	73	80	27
2	76					67		84	12
3	74	75	67	63	67	71	82		15
4	88	82	75	71	67	67		73	24
5	92	84	79	73	79	84		106	21
6	75	64	60		57	63	60		24

Rats nos. 1-5 in 11% CO_2.

Rat no. 6 in 20% CO_2.

* Values expressed as cubic centimeters of oxygen consumed per 100 square centimeters body surface per hour.

Surface area calculated by Lee's formula: $s = 12.44 \times (w)^{2/3}$

C. Acid-base Balance of Blood Resulting from Acute and Chronic Exposure to Carbon Dioxide. The nature of the shift in the acid-base equilibrium resulting from both sudden and prolonged exposure of rats to CO_2 is shown in table 2. Since pentobarbital depresses respiratory activity, a respiratory acidosis characterizes all of the control determinations made in room air. These abnormal control levels were considered adequate for comparative purposes since we were interested primarily in the degree to which the rat could compensate for the acidosis produced when exposed continuously to 11% CO_2 for a period of 2 weeks. It will be noted that the plasma pH of rats which have been exposed to 11% CO_2 and 21% O_2 for 17 days is in the same range as that found after one-half hour exposure even though the total CO_2 content is greatly increased. These results indicate that acclimatization, in so far as it occurs, is not due to a reëstablishment of a normal level of pH.

D. Effects of Various Concentrations of Carbon Dioxide on the Duration of Action and Toxicity of Pentobarbital. In the determinations of the acid-base balance of the blood during exposure to CO_2, it was noted that high concentrations of CO_2 in the atmosphere prolonged the action of pentobarbital. This action of CO_2 was therefore investigated in the following experiments.

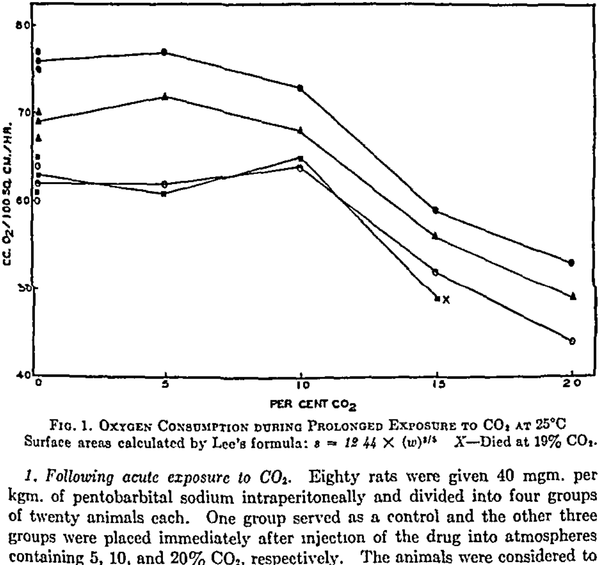

FIG. 1. OXYGEN CONSUMPTION DURING PROLONGED EXPOSURE TO CO_2 AT 25°C
Surface areas calculated by Lee's formula: $s = 12.44 \times (w)^{2/3}$ X—Died at 19% CO_2.

1. Following acute exposure to CO_2. Eighty rats were given 40 mgm. per kgm. of pentobarbital sodium intraperitoneally and divided into four groups of twenty animals each. One group served as a control and the other three groups were placed immediately after injection of the drug into atmospheres containing 5, 10, and 20% CO_2, respectively. The animals were considered to have recovered when they could assume and retain the upright position. The data are expressed in figure 2 as number of animals recovered plotted against time. The few deaths that occurred were in animals with preëxistent respiratory infections. Since no deaths occurred in animals without respiratory infections, the rats that survived may be treated statistically as homogeneous groups. Whereas 5% CO_2 had no significant effect on recovery time ("t" between 0 and 5% = 0.529), a definite delay occurred with 10% CO_2 ("t" between 5 and 10% = 3.83) and a marked prolongation was noted with 20% CO_2 ("t" between 10 and 20% = 4.5). Two other series of rats, not shown in figure 2, gave results which were qualitatively similar although the exact time relationships were not identical.

TABLE 2

Acid-base balance of blood of rats exposed to 11% CO_2*

TIME IN CO_2	PER CENT CO_2	V_c	pH_s	VOLUME PER CENT CO_2
0	0	30	7.36	84.0
		42	7.21	82.8
		42	7.26	78.4
Average		38	7.28	81.7
½ hr.	12	38	7.09	91.4
		43	7.09	91.4
		38	7.08	89.6
Average		40	7.09	90.8
4 hrs.	11	40	7.25	114.3
		45	7.10	100.1
		37	7.07	96.7
		37	7.09	104.7
Average		40	7.13	104.0
20 hrs.	11	44	7.14	120.3
		45	7.17	120.3
		40	7.39	145.0
		42	7.16	123.6
Average		43	7.22	127.3
17 days	11	39	7.10	145.8
		40	7.14	146.2
		41	7.09	128.5
		38	7.19	142.8
Average		40	7.13	140.8

* Anesthetized with sodium pentobarbital 45 mg. per kg. intraperitoneally.

The increased sleeping time could result from an interference with the mechanism of detoxication of the barbiturate or from an additive or potentiating action of the drug and CO_2. To obtain evidence relating to this question, three groups of twenty animals each were injected as outlined above. Group A served as a control and groups B and C were placed in an atmosphere containing 20% CO_2. After six hours, when 60% of group A had recovered, group B was removed from the CO_2 while group C was allowed to remain in the chamber. The mean recovery times of the groups were as follows: Group A, 5.6 hours ($\sigma = \pm 1.5$), Group B, 8.3 hours ($\sigma = \pm 1.4$), Group C, 13 hours ($\sigma = \pm 3.5$). Assuming that all the effects of CO_2 are completely and rapidly reversible, sixty per cent of group B should have recovered immediately if detoxication of the barbiturate had proceeded at the same rate as in group A. However, since only 15% of group B recovered immediately and the mean recovery time of the remaining animals in this group was two to three hours longer than in group A ("t" between B and A = 5.9), it seems clear that during the period in which the animals had been in CO_2 the detoxication of the barbiturate was proceeding at a slower rate.

The depressant action of CO_2 is shown clearly in the further prolongation of sleeping time in group C ("t" between means of B and C = 4.75), since none of this group recovered until two hours after group B had been removed, and some individuals of the group were still narcotized when removed after twenty one hours.

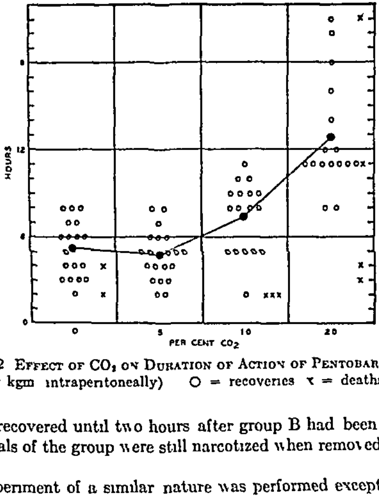

FIG. 2. EFFECT OF CO_2 ON DURATION OF ACTION OF PENTOBARBITAL

(40 mgm. per kgm. intraperitoneally) ○ = recoveries, x = deaths, ● = average sleeping time.

Another experiment of a similar nature was performed except that the animals were given 75 mgm. per kgm. of pentobarbital, which is approximately the LD_{50} (11). Contrary to what might have been predicted from the results of the experiments with smaller doses, no additive toxicity could be demonstrated, even with 20% CO_2 although the recovery time of the survivors of these experiments was prolonged in CO_2 as in the experiments cited above. The mortality percentages in the four groups (20 animals each) were: control—60%, 5% CO_2—30%, 10% CO_2—35%, 20% CO_2—55%.

A 25% prolongation of sleeping time was observed in 3 rabbits given 55 mgm.

per kgm. of amytal intravenously, and immediately placed in an atmosphere containing 20% CO_2, the mean sleeping time increasing from 3.75 hours ($\sigma = \pm 0.3$) to 4.75 hours ($\sigma = \pm 0.5$). Although too few animals were used for the results to be significant in themselves, these facts concerning the intravenous administration of the barbiturate support the findings on the rat and indicate, furthermore, that the prolonged sleeping time observed in the rat is not due to delayed absorption of the drug from the peritoneal cavity.

2. During chronic exposure to CO_2. After exposure to 10% CO_2 for seven days, 16 rats (group C) were given pentobarbital (40 mgm. per kgm.) and replaced in the same concentration of CO_2. The sleeping time was compared with that observed in two control groups of 16 rats each, group A receiving the barbiturate only, and group B placed for the first time in 10% CO_2 after the barbiturate had been administered. The mean sleeping time of group A was three hours ($\sigma = \pm 1.4$), while the mean sleeping times of groups B and C were 4.9 hours ($\sigma = \pm 2.3$) and 5.1 hours ($\sigma = \pm 2.4$), respectively. Since the "t"'s between the means of groups A and B and A and C were 2.48 and 2.74, respectively, and no significant difference exists between the means and variances of B and C, it seems clear that no tolerance to this narcotic action of the 10% CO_2 had been acquired by previous exposure for one week when tested by this method.

TABLE 3

Effect of CO_2 on liver function

	NUMBER OF ANIMALS*	AVERAGE RETENTION OF DYE AFTER			
		5 min	10 min.	15 min.	20 min.
		per cent	*per cent*	*per cent*	*per cent*
Controls . . .	6	45	23	11	0
After sudden exposure to CO_2. . .	5			23	15
After chronic exposure to CO_2	3			17	11

* The same rabbits were used for all determinations.

E. Effect of Carbon Dioxide on Liver Function. In order to obtain further evidence relating to the mode of action of CO_2 in prolonging the action of pentobarbital, bromsulfalein liver function tests were made on six rabbits during both sudden and prolonged exposure to concentrations of 10 to 15% CO_2. It will be noted (table 3) that all the dye had been removed from the blood of the controls within twenty minutes after injection. However, after acute exposure to CO_2, 15% of the dye was still present in the blood twenty minutes after the injection, and during chronic exposure 11% was still present. It is therefore apparent that such concentrations of CO_2 interfere with liver function and that there is little acclimatization to this action of CO_2.

The interference with liver function could result from a direct depression of the activity of the liver cells or from an interference with the blood flow through that organ. To obtain evidence relating to this question, 56 rats were given 45 mgm. per kgm. of pentobarbital sodium intraperitoneally and divided into two groups of 28 rats each. One group served as a control and the other was placed immediately after injection into an atmosphere containing 10% CO_2. One-half of the rats in each group then received 15 mgm. per kgm. of sodium nitrite subcutaneously at hourly intervals until a total of 60 mgm. per kgm. had been given. It was found that the nitrite alone had no effect on the sleeping time (average: 5½ hours ($\sigma = \pm 1.4$) with or without nitrite). However, the nitrite practically abolished the usual CO_2 effect on the duration of the narcotic action of pentobarbital, reducing the sleeping time from an average of 10 hours ($\sigma = \pm 1.8$) to only 7 hours ($\sigma = \pm 2.5$). Since the prolongation of sleeping time in the presence of 10% CO_2 ("t" between 0 and 10% CO_2 = 7) has been reduced by the presence of a vasodilator to an amount that can not be considered significant ("t" between 0% CO_2 and 10% CO_2 plus nitrite = 1.77), these results indicate that circulatory changes play the major rôle in prolonging the action of pentobarbital.

DISCUSSION. The results on the acute toxicity of CO_2 for short periods of exposure are in accord with the observations of Prausnitz (3) who demonstrated that laboratory animals are not affected significantly by acute exposure to 10% CO_2, and that a short exposure to 25 to 30% CO_2 produces a deep narcosis without appreciable after-effects. We have found, however, that concentrations of 20% and above are usually lethal within 96 hours of continuous exposure when the animals are placed immediately in these concentrations of the gas However, concentrations of 20 to 25% CO_2, when gradually attained, are tolerated for nearly thirty days, indicating that some degree of acclimatization may be acquired to the higher concentrations of CO_2.

This acclimatization is not due to a reëstablishment of a normal pH, a fact which is in accord with the results of Miller (7) who demonstrated that dogs exposed to 1.5 to 5% CO_2 for one to four weeks developed a mild uncompensated acidosis. In his experiments, the CO_2-combining power was reduced, indicating the existence of a metabolic acidosis.

Gesell and others (12, 13) report that short exposure to moderately high concentrations of CO_2 decreases oxidations, and therefore oxygen consumption. The data presented here support these observations since the oxygen consumption is reduced by CO_2 concentrations of 10 to 20% during the first few hours following sudden exposure. However, in the rat, the process of acclimatization is associated with a reestablishment of the normal oxidative level since the total oxygen consumption returns to the normal level within 24 hours of exposure to 10% CO_2. Moreover, if the concentrations of CO_2 are attained gradually, no reduction in oxygen consumption occurs until definitely narcotic concentrations are obtained (15% and above)

If we accept the current theories which relate narcosis to diminished oxidations in the central nervous system, it is necessary to assume that certain tissues of the body are more susceptible to the depressant effect of CO_2 on oxidations than are those of the central nervous system, since 10% CO_2 reduces the total oxygen uptake to 75% of normal, yet fails to induce a significant degree of narcosis. Furthermore, it seems probable that it is these same tissues which are involved primarily in the process of acclimatization, since 10% CO_2 effects the same prolongation of sleeping time after pentobarbital in the unacclimatized as in the acclimatized rat (one which has regained a normal level of total oxygen uptake during prolonged exposure to CO_2).

This increased duration of action of pentobarbital during both sudden and prolonged exposure to 10% CO_2 may be related to a decrease in liver function. Results obtained by the bromsulfalein test support the view. Since this increase may be practically abolished by the administration of a vasodilator, sodium nitrite, and since the inhalation of CO_2 produces an acidosis with a resulting vasoconstriction which is only slightly, if at all, compensated after prolonged exposure, it is most probable that both the increased sleeping time after pentobarbital and increased retention of the dye are due to a reduced blood supply to the liver. Moreover, this suffices to explain the failure of 5% CO_2 to affect the duration of action of pentobarbital, since Grollman (14) has shown that CO_2 does not affect the circulatory system significantly until a concentration of approximately 6% is reached.

SUMMARY

The acute and chronic toxicities of carbon dioxide have been compared and certain factors concerned in its narcotic action have been investigated.

1. The maximal tolerated concentration of CO_2 for the albino rat after sudden exposure is approximately 15%. However, when the final concentration of CO_2 is attained gradually over several days, the maximal tolerated concentration is approximately 23%. Likewise, a greater degree of depression is produced by sudden exposure to a given concentration of CO_2 than by prolonged exposure to the same concentration, indicating that the rat is capable of a certain degree of acclimatization.

2 Following sudden exposure to 11% CO_2, there is a temporary decrease in the total oxygen consumption of the rat. This reaches a level 15 to 25% below normal within two to four hours and slowly returns to the normal range within twenty-four hours of continued exposure. When the rat is placed in atmospheres with gradually increasing concentrations of CO_2, the oxygen consumption remains within normal limits until concentrations of CO_2 greater than 10% are attained.

3. During exposure to 11% CO_2 for thirty days, the plasma pH falls to a level as low as 7.09 in the first half hour and remains below 7.15 during the remainder of the period. The CO_2 content of the blood gradually increases to 130 to 148 volumes %.

4. A marked reticulocytosis occurs in the rat during prolonged exposure to 11% CO_2, but no corresponding increase in the number of leukocytes or erythrocytes is observed.

5. The sleeping time of normal rats anesthetized with pentobarbital is prolonged in atmospheres containing 10 or 20% CO_2. Similar results are obtained with rats previously exposed to 10% CO_2 for one week. Evidence is presented which suggests that CO_2 delays the detoxication of pentobarbital by diminishing the blood flow through the liver as a result of the splanchnic vasoconstriction.

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SERIAL DETERMINATIONS OF CARDIAC OUTPUT (BALLISTOCARDIOGRAM) AND ELECTROCARDIOGRAM IN NORMAL MAN AFTER THE INTRAVENOUS ADMINISTRATION OF PURIFIED CARDIAC GLYCOSIDES[1]

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The mode of action of digitalis in both the failing and the normal circulations of man remains controversial (1–4) and the circulatory effects of the purified digitalis glycosides have but recently been investigated (5–8). Many of the previous observations have been made on animals, or the excised organs of animals. Often these data have not been applicable to man (6, 9, 10). Additional observations of a physiologic nature in man seemed desirable.

This investigation had two purposes. First, to determine the effects produced by several purified cardiac glycosides on four components of the normal circulation, namely cardiac output, electrocardiogram, cardiac rate, and arterial blood pressure. Second, to compare the three glycosides, lanatoside C, digoxin, and digitoxin with regard to their effects on these circulatory components. These three drugs are of known chemical structure, available in purified crystalline form and, therefore, superior to preparations of digitalis, the composition of which is not only unknown but variable (8).

In the present study we were interested particularly in the primary effects of the glycosides: the time of their onset, and their differentiation from secondary effects. In order to achieve these aims it was decided to administer the glycosides intravenously in single therapeutic doses. This eliminated the problem of differences in absorption and produced typical, full effects within a few hours. Frequent serial determinations were now possible throughout the entire period of the response, making differentiation between primary and secondary effects readily attainable. Such a differentiation may be difficult or impossible from data obtained at intervals of many hours or days, when effects have become established. The observations were continued well past the time at which therapeutic effects appeared when similar amounts of the drugs were administered to patients with congestive heart failure.

METHODS. The subjects were hospitalized convalescent adults without heart disease and with normal circulations and normal electrocardiograms. Cardiac output was determined frequently and easily by means of a ballistocardiograph.[2] Starr, Rawson, Shcroeder and Joseph (11), Starr and Schroeder (12), and Cournand, Ranges and Riley (13) have demonstrated the accuracy of this method. In the calculation of stroke volume we have employed the correction factor of plus 18.5% suggested by Cournand *et al.* (13). Standard *limb-lead electrocardiograms were recorded simultaneously with the ballistocardiograms.* Heart rate was obtained from the electrocardiograms. A single observation consisted of a simultaneous ballistocardiogram and electrocardiogram followed immediately by a determination of the arterial blood pressure (auscultatory method using mercury manometer) and a count of the heart rate for a full minute. All observations were made with the subjects in the same supine position on the ballistocardiograph. At least two hours had elapsed between the last meal and each observation. A rest period of thirty minutes or more preceded all determinations. After at least two control observations the overlying skin was anaesthetized with 2% novocaine and the glycoside was injected intravenously. Frequent observations were then made· four to five in the first hour, eight to ten in the first three hours, ten to twelve in the first day, and thereafter daily or every other day for two days to three weeks.

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[2] For generously placing this apparatus at their disposal, the authors thank Doctor Homer Smith of the Department of Physiology, New York University College of Medicine.

Approximately two hours after administration the glycosides had usually induced definite changes in the electrocardiogram and maximum slowing of cardiac rate. The heart rate was now returned to its "initial" level by the intravenous injection of 0 3 mgm. to 0 6 mgm. of atropine sulfate, and observations were continued. This procedure was carried out in six subjects; two had previously received lanatoside C, two digoxin and two digitoxin.

RESULTS. Nine subjects received lanatoside C, nine digoxin, and ten digitoxin given as digitaline Nativelle (a commercial preparation considered to contain at least 90% crystalline digitoxin).[3] The doses of lanatoside C varied from 1.5 mgm. to 2.5 mgm., of digoxin from 1.2 mgm. to 2.5 mgm., and of digitaline Nativelle from 0.72 mgm. to 1.8 mgm. These doses usually did not cause definite symptoms or signs of digitalis toxicity, though slight anorexia and a sense of "uneasiness" were noted by some of the subjects six to ten hours after the injection of the larger doses. One subject received lanatoside C twice, in dosage of 2.0 and 1.8 mgm. Six subjects received two different glycosides, administered in equal gram molecular amounts. The second glycoside was given only after a lapse of two weeks or more, and when the electrocardiographic changes caused by the first drug had disappeared.

In five of the subjects control observations were performed with an equal volume of physiologic salt solution substituted for the glycoside. In one other subject 7% alcohol in physiologic salt solution was substituted. Some glycosides are dispensed in alcoholic solution which at the time of injection was 7 to 8%.

Electrocardiographic changes. The glycosides usually induced electrocardiographic effects but occasionally none were seen following doses which in other subjects caused definite changes. The changes induced were slight and when fully developed were of the same type and magnitude for all three glycosides. The most frequent alteration was a decrease in the amplitude of the *T*-wave in one or more leads (figs. 1, 2). Inversion of the *T*-wave was infrequent. Occasionally the *S-T* segment became slightly depressed. The *P-R* interval usually did not change, but twice it increased to 0.22 second, once to 0.21 second and once to 0.41 second. Electrical systole ("*QT*") and *QRS* were not significantly affected.

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[3] *For generous supplies of the glycosides used in their investigation,* the authors wish to thank Sandoz Chemical Works, Inc (lanatoside C), Burroughs Wellcome and Company, Inc. (digoxin), and Laboratoire Nativelle, Paris (digitaline Nativelle).

Lanatoside C and digoxin induced these changes with approximately equal rapidity (table 1; figs. 1, 2). Initial changes usually appeared within ten to fifteen minutes, maximum effects in one to six hours. Regression toward normal was definite in twenty-four to thirty-six hours and often began as early as eight to ten hours after injection. A complete return to normal required three to six days, occasionally longer (table 1).

After digitaline Nativelle, electrocardiographic changes developed and regressed more slowly (table 1; fig. 2). Initial effects usually appeared after one to one and a half hours, and only twice before thirty minutes. On this occasion slight changes occurred sixteen minutes after 0.72 mgm., the smallest dose given any patient. Maximum effects were reached in two to nine hours. Regression toward normal usually began on the second or third day, but in three instances within seven to nine hours. Minor deviations persisted as long as two to three weeks.

This difference in the development of electrocardiographic changes was most apparent when equal gram molecular amounts of two glycosides were given to the same subject (fig. 2). Differences in the individual molecules now accounted for the differences in action.

Hemodynamic effects. The analysis of the circulatory changes produced by the glycosides is limited to the primary effects, i.e., to changes occurring within the first two to three hours. It was during this period that electrocardiographic and circulatory effects developed in the normal subject and that therapeutic effects appeared when similar amounts of the glycosides were given to patients with congestive heart failure. Moreover, this was a single observation period and during it the most adequately controlled state was achieved.

Physiologic salt solution and 7% alcohol in physiologic salt solution usually had little or no effect (fig. 3). When changes did occur, the cardiac rate fell slowly, accompanied by a small increase in stroke volume and a small decrease in minute output (table 2). These changes were attributed to the prolonged rest.

The circulatory changes induced by the cardiac glycosides were usually small; never more than moderate (figs. 4, 5, 6; tables 3, 4, 5). When fully developed, their magnitude and pattern were the same following each glycoside (tables 3, 4, 5). Almost as frequent, however, was the absence of any hemodynamic change (fig. 7). This was noted with each glycoside and appeared to be not related to the amount of drug administered.

Typical, full responses (figs. 5, 6, 7) consisted of a prompt, at times considerable, decrease in heart rate; a slight to moderate increase in stroke volume; a small but consistent rise of the arterial blood pressure, largely of the systolic level and very little of the diastolic. The decrease in heart rate was proportionately greater than the compensating increase in stroke volume. As a result, the minute output decreased, but only slightly, and with it the cardiac index and the output per minute per pound. At times, the increase in stroke volume counterbalanced the decrease in heart rate and the minute output remained unchanged.

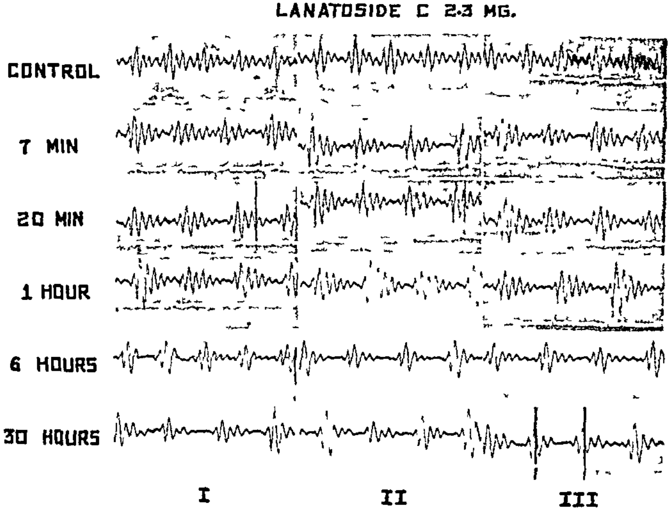

FIG. 1. SIMULTANEOUS BALLISTOCARDIOGRAPHIC AND ELECTROCARDIOGRAPHIC EFFECTS INDUCED BY LANATOSIDE C 2.3 MGM. INTRAVENOUSLY

The upper black tracing is the ballistocardiogram, the lower white the electrocardiogram with the *three* leads in order. Seven minutes after injection cardiac slowing with beginning depression of *T* waves; twenty minutes after injection considerable depression of *T* waves; *no further electrocardiographic* changes after one and six hours; next day beginning return toward normal. Increase in amplitude of ballistic complexes as cardiac rate slowed.

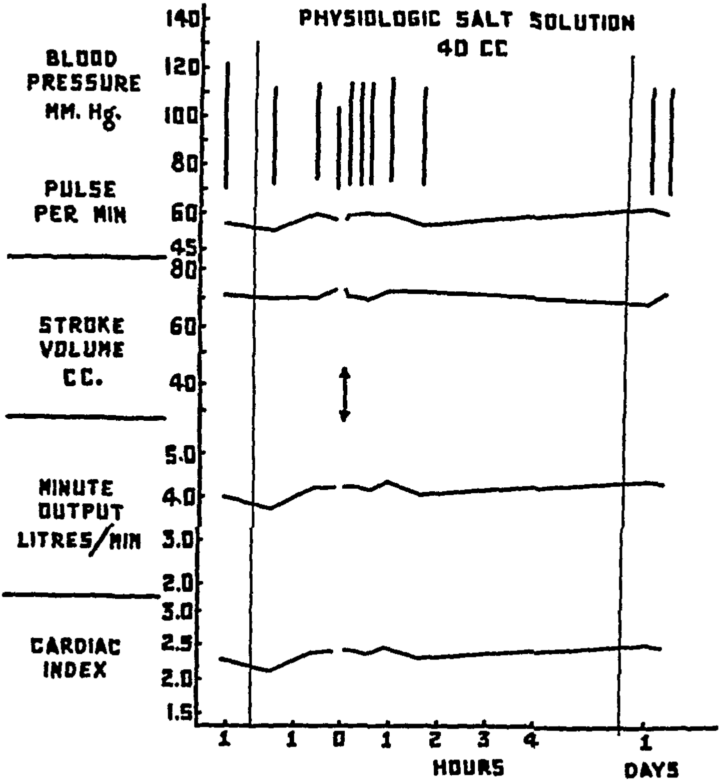

Fig 3 Failure of Physiologic Salt Solution to Produce Significant Circulatory Changes

(vertical double arrow) indicates the intravenous
e the day of the experiment, other days falling
equal percentage changes in all functions cause
equal fluctuations in all lines

TABLE 2

Circulatory effects of physiologic salt solution—administered intravenously

SUBJECT (SEX, COLOR, AGE)	DOSE	HEART RATE PER MIN			STROKE OUTPUT (CC)			MINUTE OUTPUT (LITERS PER MIN)			ARTERIAL BLOOD PRESSURE (MM Hg)		
		Control	After salt	% change	Control	After salt	% change	Control	After salt	% change	Control	After salt	% change
	cc												
Z B (*F*, *n*, *23*)	*20*	80	75	−6 2	41 5	43 8	+5 5	3 31	3 28	−0 9	102/62	108/66	+5 9/ +6 5
H T (F, c, 20)	30	72	67	−7 0	35 7	41 0	+14 8	2 57	2 79	+8 6	100/60	98/50	−2 /−16 7
J S (M, c 50)	38	52	48	−7 7	72 5	76 6	+5 7	3 77	3 55	−5 8	114/72	110/68	−3 5/ −5 5
H C (M, w, 46)	40	58	56	−3 4	73 0	72 5	−0 7	4 23	4 06	−4 0	104/70	116/74	+11 5/ +5 7
C E (*M* *w*, *21*)	*40*	*79*	*60*	−*24 0*	49 7	49 8	+0 2	3 92	2 98	−24 0	120/70	104/68	−9 7/ −2 9
J G (M, w, 44)	40	60	52	−13 3	59 3	62 4	+5 2	3 56	3 24	−9 0	114/76	106/70	−7 0/ −7 9
L L (F, c 21)	*40 8	72	76	+5 6	43 3	44 1	+1 8	3 12	3 3	−5 8	122/74	122/80	0 / +8 1
Mean ± standard error				−8 0 ±3 4			+4 6 ±1 9			−4 2 ±3 7			−0 7 ± 2 8/ 1 8 ± 3 4

* Seven per cent alcohol in physiologic salt solution

The decrease in heart rate usually occurred promptly, within ten minutes of the injection This was in contrast to the gradual cardiac slowing sometimes observed after salt solution As the heart slowed the arterial blood pressure usually rose Both of these changes rapidly became maximal, then persisted or

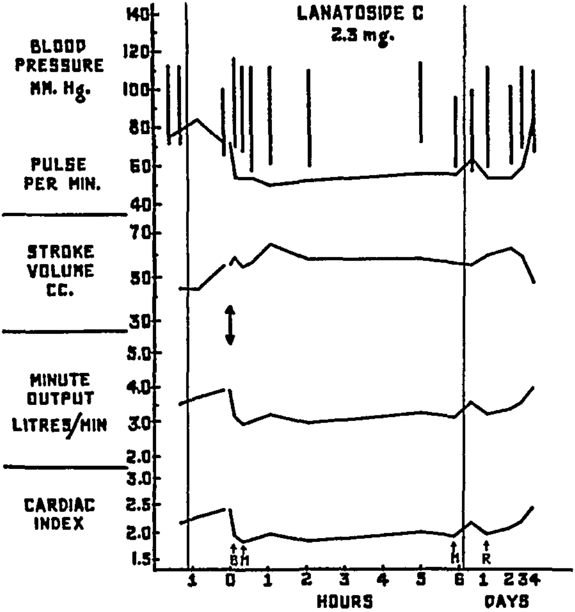

FIG 4 CIRCULATORY EFFECTS INDUCED BY LANATOSIDE C 2 3 MGM INTRAVENOUSLY
Heart rate fell from 75 to 50 per minute, arterial pressure rose from 100/66 to 116/70 mm Hg stroke volume increased from 55 to 65 cc minute output fell from 3 95 to 2 96

the letters

slowly regressed throughout the observation period Small doses of atropine readily returned the heart rate to its initial value without affecting significantly the arterial blood pressure

As the heart slowed, stroke volume increased but its maximum change usually developed more slowly, requiring forty five to ninety minutes Only occasionally did the stroke volume decrease, and then but slightly

There appeared to be a definite relationship between cardiac rate and cardiac output. When a glycoside decreased the heart rate, stroke volume increased and minute output tended to fall (figs. 4, 5); when the heart rate did not change, cardiac output remained unaltered (fig. 7).

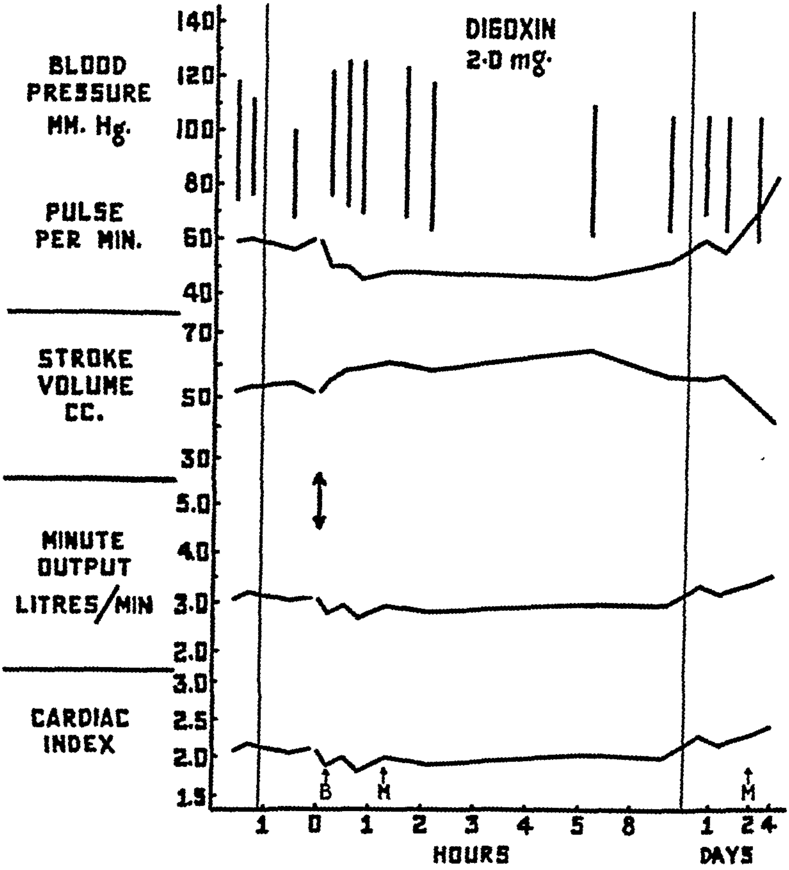

FIG. 5. CIRCULATORY EFFECTS INDUCED BY DIGOXIN, 2.0 MGM. INTRAVENOUSLY
Heart rate fell from 60 to 45 per minute, arterial pressure rose from 100/68 to 126/72 mm. Hg, stroke volume increased from 51.5 to 61 cc., minute output fell from 3.08 liters to 2.66 liters and cardiac index from 2.1 to 1.8.

The electrocardiographic and hemodynamic effects were not always related to each other. Following lanatoside C and digoxin the two changes often appeared concomitantly (figs. 4, 5) but this was not always so (fig. 7). Following digitaline Nativelle the dissociation between the two effects was readily apparent (fig. 6). In a typical instance (fig. 6) electrocardiographic changes did not appear before two hours, but within ten to twenty minutes the heart rate had fallen from fifty-four to forty-four per minute, the arterial blood pressure had risen from 120/84 to 142/94, the stroke volume had increased 6 cc , and the minute output had decreased from 4 to 3 5 liters Contrariwise, typical electrocardiographic changes at times followed each of the glycosides without significant alteration in the circulation (fig 7)

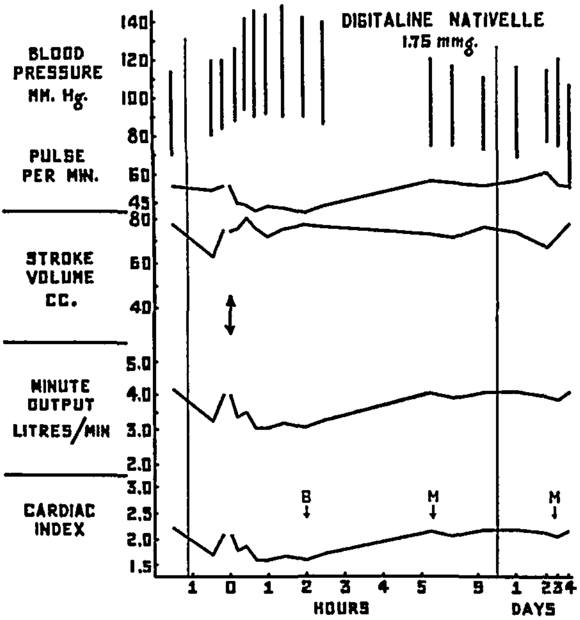

Fig 6 Circulatory Effects Induced by Digitaline Nativelle, 1 75 mgm Intravenously

Heart rate fell from 54 to 44 per minute, blood pressure rose from 120/84 to 142/94 mm Hg stroke volume increased from 74 to 80 cc but minute output fell from 4 0 to 3 5 liters and cardiac index from 2 1 to 1 8 Note dissociation of the onset of hemodynamic and electrocardiographic effects

Release of vagal tone The slowing of the heart rate raised this question

To what extent were the established electrocardiographic and hemodynamic effects the result of the slower cardiac rate?

When the heart rate was returned to its *control* value by atropine, the electrocardiographic effects persisted, or even progressed. There was a tendency for the amplitude of the *T* waves to decrease further and for the *S-T* segments to become slightly depressed. The associated hemodynamic changes were more variable (table 6). Most often, the stroke volume decreased sharply and became smaller than the stroke volume of the control period. As a result, the

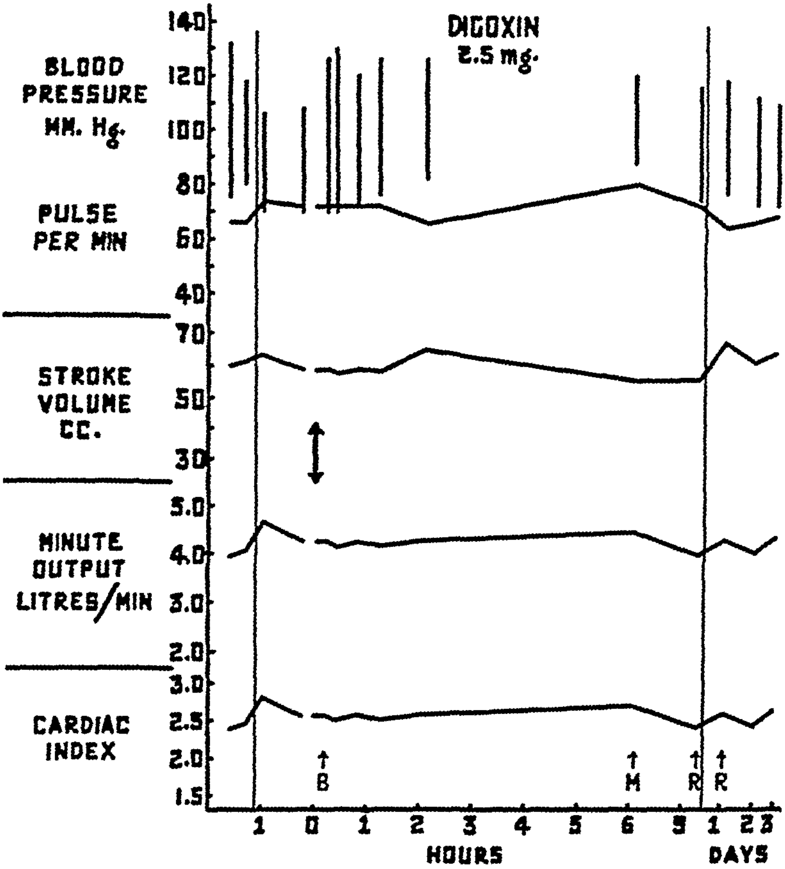

FIG. 7. CIRCULATORY EFFECTS INDUCED BY DIGOXIN, 2.5 MGM. INTRAVENOUSLY

In this subject circulatory changes were practically absent. Cardiac output and cardiac index remained unchanged. Cardiac rate and stroke volume showed slight and counter-[illegible] after [illegible] hours. Blood pressure rose promptly from 108/70 to 130/70 mm. [illegible] changes appeared promptly, dissociating from the circulatory effects.

minute output also remained below that of the control period and continued to approximate that lower value to which it had fallen after the administration of the glycoside (table 6, fig 8). When atropine caused the heart rate to increase beyond the initial value, the minute output now exceeded the control output, even though the stroke volume decreased still further.

TABLE 3

Circulatory effects of lanatoside C—administered intravenously

SUBJECT (SEX, COLOR, AGE)	DOSE	HEART RATE PER MIN			STROKE OUTPUT (cc)			MINUTE OUTPUT (LITERS PER MIN)			ARTERIAL BLOOD PRESSURE (MM Hg)		
		Control	After glycoside	% change	Control	After glycoside	% change	Control	After glycoside	% change	Control	After glycoside	% change
	mgm												
A W (F, w 23)	1.5	82	66	−19.5	50.8	54.2	+6.7	4.18	3.51	−16.0	102/62	108/68	+5.9/ +9.7
M H (F, c 46)	1.5	52	45	−13.4	50.9	53.1	+4.3	2.64	2.39	−9.5	98/70	114/72	+16.3/ +2.9
L L (F, c 21)	1.6	69	53	−23.2	43.0	50.5	+5.2	3.24	2.76	−14.8	116/74	130/80	+12.0/ +8.1
R W (M c 34)	1.8	69	48	−29.8	65.4	81.4	+24.5	4.52	3.94	−12.8	100/60	112/68	+12.0/+13.3
R W (M c, 34)	2.0	65	47	−27.7	77.8	88.9	+14.3	5.07	4.07	−19.7	106/70	116/68	+9.4/ −2.9
J B (M, w, 45)	2.0	72	75	+4.2	62.0	71.0	+14.7	4.46	5.21	+16.8	116/88	134/90	+15.5/ +2.3
F G (M, w, 53)	2.0	70	65	−7.1	58.2	58.8	+1.0	4.07	3.64	−10.6	114/70	118/74	+3.5/ +5.7
N G (M, w, 45)	2.2	72	72	0	69.3	70.7	+2.0	4.98	5.09	+2.2	114/74	136/82	+19.3/+10.8
J W (M w, 33)	2.3	73	50	−30.6	54.9	64.5	+17.5	3.95	3.22	−18.5	100/66	116/70	+16.0/ +6.1
A R (M, c 56)	2.5	60	41	−18.0	68.8	77.0	+11.9	3.44	3.16	−8.2	102/68	140/72	+37.2/ +5.9
Mean ± standard error				−16.5 ±3.9			+10.2 ±2.4			−9.1 ±3.5			+14.7±2.9/ +6.2±1.5

TABLE 4

Circulatory effects of digoxin—administered intravenously

SUBJECT (SEX, COLOR, AGE)	DOSE	HEART RATE PER MIN			STROKE OUTPUT (cc)			MINUTE OUTPUT (LITERS PER MIN)			ARTERIAL BLOOD PRESSURE (MM Hg)		
		Control	After glycoside	% change	Control	After glycoside	% change	Control	After glycoside	% change	Control	After glycoside	% change
	mgm												
M H (F, c, 46)	1.2	61	51	−16.4	48.6	54.0	+11.1	2.96	2.73	−7.8	106/68	110/72	+3.8/ +5.9
K W (F, c 33)	1.5	90	96	+6.7	34.7	38.0	+9.5	3.12	3.64	+16.6	106/70	126/76	+18.9/ +8.6
J G (M w 44)	1.57	56	48	−14.3	66.0	70.0	+4.5	3.69	3.30	−10.6	110/78	114/80	+3.8/ +2.6
H T (F, c 20)	1.6	66	52	−21.2	35.1	38.2	+8.8	2.31	1.98	−14.3	112/70	124/64	+10.7/ −8.6
J S (M, c, 50)	1.8	60	56	−6.7	61.0	63.4	+3.9	3.66	3.55	−3.1	94/62	118/76	+2.3/+16.1
K C (F, w 44)	2.0	60	45	−25.0	51.4	59.0	+14.8	3.08	2.66	−13.6	100/68	128/76	+28.0/+11.7
H S (M, w, 20)	2.25	61	50	−18.1	68.6	76.3	+11.2	4.20	3.82	−9.1	112/68	122/62	+8.9/ −8.8
A R (M, c, 56)	2.5	54	48	−11.1	62.9	64.3	+2.2	3.40	3.09	−9.1	112/66	138/72	+23.2/ +9.1
F J (M c 53)	2.5	72	72	0	58.7	57.6	−1.9	4.22	4.15	−1.7	109/70	130/70	+20.4/ 0
Mean ± standard error				−11.8 ±3.4			+7.1 ±1.6			−5.8 ±3.1			+13.1±3.1/ +4.1±2.9

TABLE 5

Circulatory effects of digitaline nativelle—administered intravenously

SUBJECT (SEX, COLOR, AGE)	DOSE	HEART RATE PER MIN			STROKE OUTPUT (CC)			MINUTE OUTPUT (LITERS PER MIN)			ARTERIAL BLOOD PRESSURE (MM Hg)		
		Control	After glycoside	% change	Control	After glycoside	% change	Control	After glycoside	% change	Control	After glycoside	% change
	mgm												
F V (F, w, 21)	0 72	76	70	—7 9	45 3	46 0	+1 5	3 44	3 20	—7 0	106/74	112/76	+5 7/ +2 7
Z B (F, w, 23)	1 0	78	71	—9 0	40 8	44 2	+8 3	3 17	3 14	—0 9	108/64	114/66	+5 5/ +3 1
A B (F, c, 39)	1 4	86	75	—12 8	48 2	50 7	+5 2	4 16	3 76	—9 6			
H T (F, c, 20)	1 5	65	52	—20 0	35 4	41 5	+17 2	2 30	2 16	—6 1	104/62	128/86	+23 1/+38 7
H C (M, w, 46)	1 5	60	54	—10 0	73 0	64 6	—11 5	4 38	3 49	—20 3	114/70	134/86	+17 5/+22 9
J G (M, w, 44)	1 52	54	49	—11 1	65 5	58 0	—11 4	3 53	2 94	—16 7	106/78	110/72	+3 8/ —7 7
J S (M, c, 50)	1 75	54	40	—26 0	74 3	76 5	+3 0	4 01	3 06	—23 6	120/84	150/94	+25 0/+11 9
F K (M, w, 41)	1 75	66	54	—18 2	75 5	74 6	—1 9	5 05	4 16	—17 6	110/60	118/68	+7 3/+13 3
M S (F, c, 22)	1 76	72	57	—20 8	43 4	49 0	+12 9	3 11	2 83	—9 0	112/68	120/74	+7 2/ +8 8
N G (M, w, 45)	1 8	79	75	—5 1	70 0	78 1	+11 6	5 52	5 85	+6 0	106/72	116/78	+9 5/ +8 4
Mean ± standard error				—14 1 ±2 1			+3 5 ±3 1			—10 5 ±2 9			+11 6±2 7/ +11 3±4 4

TABLE 6

Circulatory changes accompanying the return of the cardiac rate to the control level when atropine was given after the effects of the previously administered glycoside had become established

SUBJECT (SEX, COLOR, AGE)	GLYCOSIDE	ATROPINE SULFATE	CARDIAC RATE PER MIN			BLOOD PRESSURE (MM Hg)			STROKE VOLUME (CC)			MINUTE OUTPUT (LITERS PER MIN)			CARDIAC INDEX		
			Control	Effect of glycoside	Effect of atropine sulfate	Control	Effect of glycoside	Effect of atropine sulfate	Control	Effect of glycoside	Effect of atropine sulfate	Control	Effect of glycoside	Effect of atropine sulfate	Control	Effect of glycoside	Effect of atropine sulfate
Lanatoside C																	
	mgm	*mgm*															
M H (F, c, 46)	1 5	0 5	52	45	52	98/70	106/64	104/64	50 9	51 8	43 4	2 64	2 32	2 26	1 7	1 5	1 45
L L (F, c, 21)	1 6	0 3	69	54	64	116/70	130/80	122/78	47 0	51 2	46 3	3 24	2 76	2 96	1 7	1 4	1 6
Dixogin																	
M H (F, c, 46)	1 2	0 3	61	51	60	106/68	110/64	106/66	48 6	53 5	46 3	2 96	2 73	2 77	1 9	1 75	1 8
J G (M, w, 44)	1 57	0 3	56	48	57	110/78	110/78	114/82	67 6	70 0	53 0	3 78	3 35	3 02	2 25	2 0	1 8
Digitaline nativelle																	
M S (F, c, 22)	1 76	0 3	72	57	72	110/78	122/74	114/72	43 3	49 5	40 2	3 11	2 62	2 89	1 9	1 7	1 8
F K (M, w, 41)	1 75	0 5	65	51	60	110/62	124/70	126/74	76 1	81 9	66 0	4 95	4 16	3 96	3 1	2 6	2 4

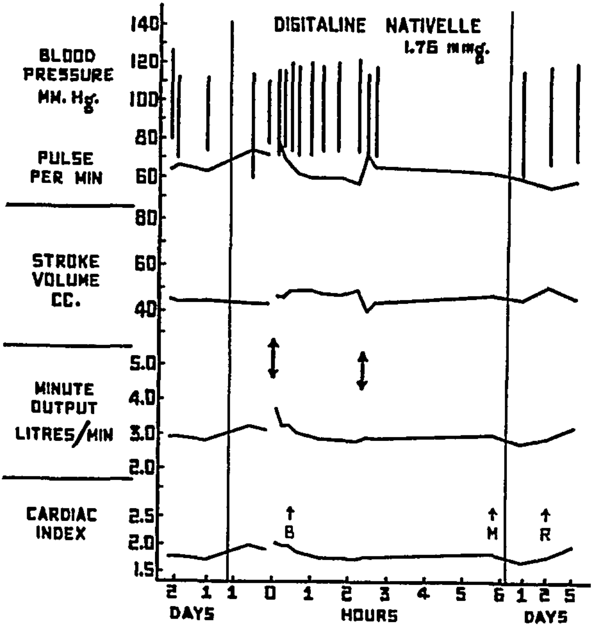

Fig 8 Circulatory Changes Induced by Digitaline Nativelle, 1.76 mgm Intravenously and by Atropine Sulfate 0.3 mgm Intravenously when Effects of Glycoside Had Become Established

The first arrow indicates injection of glycoside, the second arrow injection of atropine

	Cardiac rate per min	Blood pressure mm Hg	Stroke volume, cc	Minute output, liters per min	Cardiac index
Control	72	110/74	43.5	3.11	1.91
Effect of digitaline nativelle	60	120/72	49.2	2.95	1.82
Effect of atropine sulfate	72	114/72	40.2	2.89	1.77

Discussion The three cardiac glycosides, lanatoside C, digoxin, and digitoxin (as digitaline Nativelle), were similar in that they produced electrocardiographic changes which, when fully developed, were of the same type and magnitude The glycosides differed from each other in the rapidity with which they induced these changes, and in the persistence of their effects This was evident when the glycosides were administered in doses of equal weight. It was particularly true when equal gram molecular amounts of the different glycosides were given, after an appropriate interval, to the same subject. This latter method amounts to the administration of equal numbers of molecules of the different glycosides. It is now possible to compare the glycosides molecule for molecule. Such a comparison is believed to give a more faithful concept of the differences between the individual glycosides than comparisons utilizing doses based on units of potency derived from biological assay. The present study does not deal with a comparison of the glycosides in terms of their equally effective therapeutic doses.

Following digitaline Nativelle electrocardiographic changes made their appearance more slowly and were more persistent than after lanatoside C and digoxin, which acted with approximately equal (and surprising) rapidity. Since the drugs were given intravenously these differences probably depend on intrinsic variations in the chemical structure or physical chemical properties of the glycosides. The relationship between these characteristics of the glycosides and their function is not explained by this study.

The doses of atropine here employed were not sufficiently large to remove completely the vagal tone. However, the persistence of the electrocardiographic effects as the cardiac rate returned to the control level or beyond, suggests that the electrocardiographic changes were cardiac rather than vagal in origin.

Since the effect on cardiac rate appeared to determine the other hemodynamic changes and since the slowing of the heart rate was of vagal origin, the circulatory effects of the glycosides seemed to depend on their vagal rather than intrinsic cardiac action. This was substantiated by the lack of relationship between the hemodynamic and electrocardiographic effects, the latter here considered indicative of an intrinsic, cardiac effect of the glycosides. (Changes in the *T* wave in the absence of change of position or of external stimuli are thought to arise from the heart itself).

The oral administration of digitalis whole leaf has been reported to produce a decrease in cardiac (minute) output (14). An evaluation of the hemodynamic effects of the intravenously administered glycosides is difficult because of the inconstancy of the results produced. Doses which on occasions induced considerable alterations failed in other subjects to cause any effect, or resulted in small changes. The typical, full effect when present was similar for the three glycosides. It consisted of a considerable decrease in cardiac rate, a small to moderate increase in stroke volume, a small decrease in minute output and a small but consistent rise in arterial blood pressure. The usual change in all functions averaged 10% to 15% of the initial value, but at times amounted to 20% to 25%, particularly in the cardiac rate. The resultant values never fell beyond the wide range of normal for any function studied.

These hemodynamic changes were similar to the most marked changes observed during the prolonged rest of the control observations with salt solution.

But the changes induced by the glycosides appeared more quickly and more frequently and tended to be of greater magnitude.

The means for the changes produced by all three glycosides in the heart rate, stroke volume, minute output and blood pressure were subjected to statistical analysis by the *t* test. The mean blood pressure change was the only mean which differed significantly from the corresponding control mean at $P = 0.05$. However, if instead of analyzing the means one examines all of the trials with both the glycosides and salt solution, there appears to be a definite tendency for the above-described pattern of hemodynamic change to occur more frequently and more clearly after the glycosides than after salt solution. The questionably significant difference between the means of the two groups may be attributed to the small sample size in the control group and to the presence therein of a single highly aberrant observation.

There was a tendency for lanatoside C to produce the most marked cardiac slowing and increase in stroke volume, for digoxin to produce but little decrease in heart rate and for digitaline Nativelle to have very little effect on stroke volume. However, the above statistical tests indicated that the only significant difference between the hemodynamic effects of the three glycosides was the failure of digitaline Nativelle to increase the stroke volume.

The temporary, small increase in arterial blood pressure which consistently followed the administration of the glycosides, was independent of the cardiac rate and occurred regardless of the effect on stroke or minute volume. This may suggest a peripheral, non-cardiac, action of the glycosides and also discounts simple blood pressure readings as indices of cardiac function.

SUMMARY

1. The cardiac glycosides, lanatoside C, digoxin and digitaline Nativelle, when administered intravenously in single therapeutic doses to normal subjects, induced small to moderate changes in the electrocardiogram and in several circulatory functions.

2. When fully developed, the type and magnitude of both the electrocardiographic and circulatory changes were essentially the same for all three glycosides.

3. The electrocardiographic changes induced by digitaline Nativelle appeared more slowly and were more persistent than those induced by lanatoside C and digoxin, which acted with approximately equal, and surprising, rapidity.

4. The typical, fully developed circulatory changes when present consisted of:

a) A prompt, often moderate decrease in heart rate, vagal in origin.
b) A rapid small rise in arterial blood pressure, chiefly systolic.
c) A slight, occasionally moderate, increase in stroke volume.
d) A slight or questionable decrease in minute output.

5. The cardiac rate appeared to determine the other circulatory changes.

6. There was no relationship between the electrocardiographic and the hemodynamic effects.

7. The circulatory effects appeared to depend principally on the vagal action of the glycosides; the electrocardiographic changes on their direct, cardiac action.

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REDUCTION OF FLUID LOSS FROM DAMAGED (BURNED) TISSUES BY A BARBITURATE[1]

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The shocking, even killing early effects of burns can often be attributed to the loss of protein rich fluid from the burned surface. Under circumstances where an abundance of blood plasma is immediately available for replacement therapy, the serious consequences of this loss can usually be prevented. On the other hand, plasma is often not immediately available, if at all. In such circumstances, it is of great importance to curtail the loss of fluid and protein from the damaged vessels. The purpose of this report is to present evidence that when tissues are damaged by heat, the resulting loss of fluid and protein from the burned surface can be reduced significantly by the systemic effect of a barbiturate, pentobarbital sodium ("nembutal") in sedative dose.

METHODS. *Animals.* In searching for a suitable experimental animal in which blistering and copious weeping of the burned surface would occur, like that in man, we examined chickens, rabbits, cats, dogs and young pigs. In none of these were we able to produce blisters or fluid loss through the skin to the extent observed in man following a burn. Of these five groups of animals the blisters and fluid loss produced in chickens were nearest like those of man. Preliminary observations on 10 young full grown female barred rock chickens and experiments on 128 rabbits constitute the basis for the conclusions arrived at in this study. In nearly all cases the rabbits were mongrel albinos. Because of the general shortage of animals it was necessary in a few cases to use grays or spotted ones. The composition of control and test groups was the same in this regard as it was with respect to age and weight.

Anesthesia. It is evident that in a study of this type inadequately considered anesthesia might mask or otherwise vitiate the results obtained. General anesthesia was avoided in the following ways. In the case of the ten *chickens*, the cerebral hemispheres were exposed under local anesthesia (1.0% procaine hydrochloride) and the surface of the hemispheres destroyed to a depth of 1 to 2 mm. with an electric cautery. Pain perception was thus prevented. Twenty eight *rabbits* that were to receive skin burns were prepared under ether anesthesia 18 to 48 hours before the burns were carried out (the control and test animals were treated alike in this regard) by transection of their spinal cords at the level of the third lumbar vertebra in order to provide anesthesia but at the same time to preserve the sympathetic nerve supply of the region to be burned. Six 8 week old *pigs* were prepared in this way also. Fifty eight *rabbits* that were to receive burns of the small intestines required only local anesthesia (1.0% procaine hydrochloride) of the abdominal wall, since intestinal burns do not give rise to pain.

Burns. In all cases two animals were prepared and burned in exactly the same manner. Hot water was used to produce the burn. The temperature and duration of the applica-

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

tion which would lead to the greatest fluid loss were determined. Following the burn, one animal chosen at random, was given barbiturate while the other was used as control.

Chickens. Feathers were plucked from the lower half of the body and legs and the lower third of the body was burned by immersion in water at 70°C. for 5 seconds. The outer layer of burned skin became loose and could be rubbed off with a rough cloth, leaving a denuded surface which was constantly wet from oozing fluid.

Rabbits and Pigs with Skin Burns. On the day of the experiment the fur of the lower half of the trunk and hind legs was removed with a sodium sulphite depilatory paste. The skin was washed well to free it from the depilatory. Each of a pair of animals was then burned by dipping the lower third of the body into water at 90° C. for 1.5 seconds, care being taken not to burn above the anesthetic area. The surface of the burned skin was rubbed with coarse sandpaper to remove the outer layer and to expose the moist surface beneath.

Rabbits with Intestinal Burns. Each animal was strapped to a light board where it remained throughout the experiment. This facilitated the burning, weighing and handling. Under local anesthesia, as described above, a mid-line abdominal incision was made and a thin rubber sheet was stitched to the wound edges. This prevented wetting of skin and fur. After considerable trial the following procedure was arrived at: The cecal end of the small intestine was found and measuring from this point 90 cm. of the small intestine were gently lifted out of the abhominol cavity. The animal was turned on its side and 80 cm. of small intestine were allowed to slip into a basin containing water at 60° C., where they remained for 5 seconds. Care was taken to avoid all tension on the gut. The intestines were then spread out on the rubber sheet, and the transudate allowed to drain away. No differences could be detected in peristalsis between control and barbitalized animals, although such differences were constantly sought.

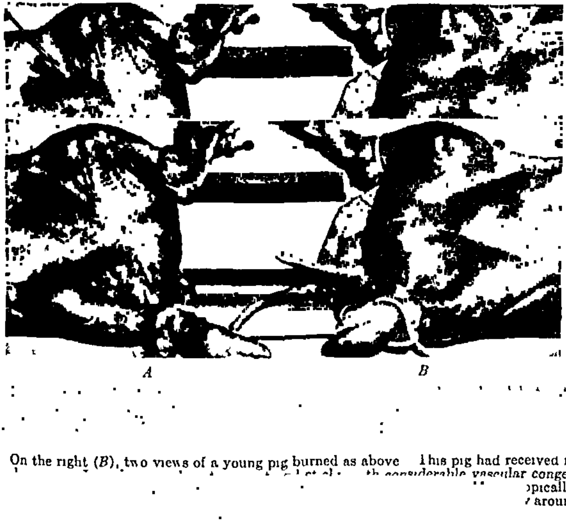
Use of the Barbiturate. In all cases, *following* the preparation and burning of the pairs of animals, one animal was chosen at random and was given intravenously a sedative but not anesthetic dose of pentobarbital sodium. The other animal served as control. The initial dose of the barbiturate was 15 or 20 mgm. per kgm. in 2.0% solution. Additional doses were given over the succeeding 4 hours so that the total quantity of the barbiturate administered amounted to about 40 mgm. per kgm. for the entire period of observation. The degree of sedation was about the same as that an average man would get from the oral administration of 0.2 to 0.25 grams of the agent.

Measurement of Fluid Loss from the Burned Surface. Care was taken to see that the fluid which escaped from the burned surface either evaporated or drained from the animal. In the cases where a rubber sheet was used, this was wiped dry before the fluid loss was measured. The fluid which escaped from the burned surface was determined by weighing the animals at hourly intervals on a balance accurate to 0.5 gram, and the loss calculated by difference. In most cases both the urethra and anus were tied off under local anesthesia, except in those cases where spinal cord transection made the use of local anesthesia unnecessary. In the majority of cases the bladder was emptied by catheterization. In the cases where the anus was not tied off, any fecal matter expelled during the course of the experiment was carefully collected and the weight loss corrected. The simultaneous study of control and test animals eliminated whatever hazard might have arisen from seasonal differences as well as day to day differences in evaporation rates due to laboratory variations in humidity, room temperature or barometric pressure. The fluid loss, followed over a four hour period, was calculated in terms of percentage of the original body weight. Corrections were made for the barbiturate solution injected, and for weight changes occurring during the intermittent blood pressure measurements.

Differences in fluid loss were grossly apparent in the skin burn experiments even to the casual observer. The skin of the animals under the barbiturate became parchment-like and was drier than the wet, sticky, puffy skin of the controls. See figure 1.

Measurement of and Correction for Fluid Loss from the Lungs. While the doses of barbiturate used were not great enough to produce more than slight if any respiratory depression, the total respiratory exchange in the controls was greater than in the animals under the barbiturate The latter group was less excitable and not inclined to hyperventilate every time some one stirred in the laboratory as was the case with the control animals Because of this difference the water loss from the lungs of two groups of normal animals had to be determined These measurements were made in 30 rabbits, untraumatized except for exposure of one carotid artery for the measurement of blood pressure and removal of blood samples Half of these animals had the barbiturate as described above, and half had none

Blood Pressure Measurement and Blood Sampling The skin of the neck of the animals was anesthetized with local anesthesia (0 2% procaine hydrochloride), a carotid artery was



On the right (*B*), two views of a young pig burned as above This pig had received no [illegible] considerable vascular conges[illegible] [illegible]opically, [illegible] around

exposed and tied off Arterial blood samples (2 0 c c) were withdrawn and heparinized for hematocrit (1 25 c c , Rourke-Ernstene) and plasma protein (Zeiss refractometer) determinations Samples were taken before the burn and at the end of the experimental period, usually 4 hours after the burn Samples of the transudate escaping from the intestinal vessels were collected and analyzed for protein by the refractometer method

Mean arterial blood pressure was estimated intermittently by the use of an 18 gauge needle thrust into the carotid artery and attached to a mercury manometer Pressure determinations were made before the burn and at intervals (about hourly) after the burn in control and in medicated animals In the case of the chickens, the exceedingly small size of their available arteries made it impossible, without resorting to near micro technic, to measure blood pressure satisfactorily This was a large factor in our decision to shift to rabbits, see "Comment" below

Body Temperature. Heating pads were used throughout these experiments and with the exception of the burned area, the animal was covered to prevent loss of heat. The body temperature was followed by placing a thermometer in the abdominal cavity through a small slit in the wall. The temperature of the control and test animals was maintained at the same level, near normal.

Comment on the Methods and Materials. After the small series of chickens had been studied, it seemed fairly clear that the effect sought for, curtailment of fluid loss from a burned surface, was present. As far as the chicken experiments were concerned, we could not be certain that the observed effect on fluid loss was not due to lowering of the systemic arterial pressure by the barbiturate used, certainly undesirable in considering possible therapeutic usefulness of the effect. Because of the difficulty in measuring accurately arterial blood pressure in chickens and because we preferred to study mammals, we shifted to rabbits.

While a barbiturate effect in curtailing fluid loss from the burned skin was demonstrated in the case of the 10 chickens and the first (28) rabbit group we suspected that the difference observed between control and test subject in the case of the rabbits at least might have been larger had it been possible for the fluid to well up from the deeper skin layers, possibly through sweat glands as may take place in man.

In the experiments reported in this paper, it was important to eliminate or circumvent two primary difficulties: (a) pain in the experimental procedure that would require general anesthesia and (b) skin structure of animals dissimilar to that of man. The anesthesia problem was minimized to the point of relative unimportance as described above. The latter problem was at least partially overcome by testing in a critical way whether or not barbiturates could curtail the fluid loss from burned intestinal blood vessels, that is, under circumstances where difficulty of escape of fluid through overlying tissues was not an interfering factor.

Six to eight week old pigs were not satisfactory for demonstrating weight loss from burns, for differences in subcutaneous fat of the body as a whole from one animal to another may greatly alter the weight, yet the low surface area suitable for burning with cord transection (at about the third lumbar vertebra) remains nearly the same. This interferes with a demonstration of true differences in fluid loss in terms of percentages of body weight. Furthermore, a relatively small area of the body surface of pigs could be burned in comparison with that possible in the case of rabbits when the spinal cord was transected at the level of the third lumbar vertebra; accordingly the fluid loss was small in proportion to the total weight of the animal, and experimental errors proportionally great. The pigs were useful, however, to demonstrate grossly drying of the burned skin following the administration of barbiturates, although they were no better than the rabbits in this regard. See the Figure.

Morphine. Since morphine is widely employed in controlling pain in burned subjects it was necessary to investigate whether or not morphine would curtail fluid loss from a burned surface as well as a barbiturate. The procedure described above for pairs of rabbits with intestinal burns was repeated with 13 pairs of rabbits where one of each pair received initially about 1.4 mgm per kgm of morphine hydrochloride intravenously; over the 4 hour experimental period about 0.7 mgm per kgm were added to the initial dose. This produced marked analgesia and definite respiratory depression. The respiratory water loss was determined in 12 unburned rabbits under morphine. This was compared with the respiratory water loss of a group of 15 normal control rabbits. These data were used to correct the morphine group and its control group so that attention might be focussed upon the fluid loss from the burned surface.

RESULTS. The data obtained are presented chiefly in the tables.

While occasionally we encountered a control animal that lost no more fluid than the animal under the barbiturate, we have never seen an animal of the latter group that lost to a significant degree more fluid than the control, although

TABLE 1

Data with standard errors of the mean

	CHICKENS, SKIN BURNS	RABBITS SKIN BURNS	RABBITS, INTESTINAL BURNS	RABBITS, RESPIRATORY WEIGHT LOSS	RABBITS, INTESTINAL BURNS	RABBITS, RESPIRATORY WEIGHT LOSS
	Lower ½ body in 70° water for 3 sec	Lower ½ body in 90° water for 1 5 sec	With water at 60°C for 5 sec	No burn	With water at 60°C for 5 sec	No burn
Control						
Number of animals	5	14	16	15	13	Same as in column 4
Sex						
Males	0	4	8	12	10	
Females	5	10	8	3	3	
Body weight (grams)	1157±70	2378±124	2193±28	2394±58	1913±91	
Four hour weight loss (percent body weight)	3 42±0 09	1 88±0 08	2 12±0 12	0 62±0 04	2 05±0 18	
Hematocrit (percent)						
Preburn		38 1±1 3	41 1±1 2	41 1±1 0	40 7±0 8	
Four hours post burn		41 1±1 7	50 9±1 4	40 6±1 2	46 1±1 7	
Plasma protein (per cent)·						
Preburn		6 19±0 16	6 30±0 15	6 23±0 15	6 12±0 24	
Four hours post burn		5 27±0 20	6 00±0 20	5 66±0 23	5 55±0 23	
Blood pressure (mm Hg)						
Preburn		102±2	114±3	117±4	113±3	
Two hours post burn		80±4	89±3	97±3	87±4	
Four hours post burn		60±5	76±4	91±2	66±6	
Pentobarbital Sodium					Morphine HCl	
Number of animals	5	14	16	15	13	12
Sex						
Males	0	7	11	12	10	10
Females	5	7	5	3	3	2
Body weight (grams)	1316±82	2351±65	2242±68	2345±74	2037±133	2012±122
Four hour weight loss (percent body weight)	2 60±0 30	1 57±0 08	1 68±0 10	0 52±0 04	1 83±0 10	0 43±0 03
Hematocrit (percent)						
Preburn		37 7±1 0	42 1±1 0	40 0±1 1	41 8±1 5	40 1±1 2
Four hours post burn		41 6±1 5	52 8±1 3	40 8±1 5	49 3±1 3	40 0±2 0
Plasma protein (per cent)						
Preburn		6 13±0 18	6 22±0 13	6 28±0 15	5 98±0 17	6 49±0 17
Four hours post burn		5 53±0 23	5 87±0 20	5 44±0 17	5 67±0 21	5 64±0 22
Blood pressure (mm Hg)						
Preburn		104±2	118±2	115±2	114±3	110±4
Two hours post burn		78±3	82±4	94±3	89±3	97±5
Four hours post burn		67±4	72±5	92±2	67±4	89±4

customarily the control lost more than the animal with the barbiturate. If the barbiturate had no real effect one would have expected random results here.

The high protein content of the fluid which leaked from the burned intestines demonstrates severe capillary damage. The percentages are as follows: 5.56 ± 0.25 for the barbiturate control group, 5.03 ± 0.20 for the animals under barbiturate, 5.17 ± 0.40 for the morphine control group and 5.12 ± 0.31 for the animals under morphine.

TABLE 2

Summary of fluid loss data

SUBJECT	CONTROL		BARBITURATE		FLUID LOSS OF CONTROL GREATER THAN THAT OF ANIMAL WITH BARBITURATE
	Number of animals used	Fluid loss* (4 hour)	Number of animals used	Fluid loss (4 hour)	
					percent
Chickens—skin burn	5	3.42 ± 0.09	5	2.60 ± 0.30	32
Rabbits—skin burn	14	1.88 ± 0.08	14	1.57 ± 0.08	20
Rabbits—intestinal burn	16	2.12 ± 0.12	16	1.68 ± 0.10	26
In order to eliminate respiratory water loss and to limit consideration to the fluid lost through the burned areas the following measurements were made of the water lost through the lungs					
Rabbits—no burn	15	0.62 ± 0.04†	15	0.52 ± 0.04	
When the above data (rabbit) are corrected by subtracting the respiratory water loss the fluid loss through the burned area is found to be as follows.					
Rabbits—skin burn	14	1.26	14	1.05	20
Rabbits—intestinal burn ..	16	1.50	16	1.16	29
The following data show that morphine has no such effect as the barbiturate·					
			MORPHINE		
Rabbits—intestinal burn	13	2.05 ± 0.18	13	1.83 ± 0.10	12‡
Rabbits—respiratory loss	15	0.62 ± 0.04†	12	0.43 ± 0.03	
Rabbits—intestinal burn corrected for respiratory loss	13	1.43	13	1.40	2

* Percent of body weight with standard error of the mean

† Data from the same group of unburned, unmedicated rabbits used as respiratory control for barbiturate and morphine groups.

‡ This disappears when the respiratory correction is made, see below

Autopsies were routinely performed. It early became apparent that the duodenal mucosa of the rabbits with skin burns that had received barbiturates were grossly much paler than the others The pathologist, Dr Benjamin Castleman, considered the duodenums of 16 animals as "unknowns" He differentiated microscopically between those showing abnormal capillary congestion and those that did not. The former were in the control group and the latter had received the barbiturate. With 16 chances for error he differentiated accurately in 12 cases, his descriptions were equivocal in 2 and in 2 they failed to agree with what might have been expected on the basis of the other experience. Considering the variability of action of the barbiturates, this is a rather striking result. It merits further study, in conjunction with the well-known tendency of burns to produce intestinal lesions.

In the case of the barbiturate studies, in both the skin and intestinal burns, essentially the same mean fluid loss values were obtained with half the experiments as with the final number; therefore since no morphine effect on fluid loss was present in 13 pairs of animals, further experiments in this direction appeared to be pointless.

DISCUSSION. *Basis for the Present Study.* It has been observed by Seeley, Essex and Mann (1936) and by Beecher, McCarrell and Evans (1942) that fluid loss from traumatized intestines is less under barbiturates than under ether. Polderman and Beecher (1941) have observed that the flow of lymph under standardized conditions is less under barbiturates than under ether. While these data are concerned with a comparison of barbiturates and ether, it seemed possible that barbiturates might also reduce the fluid loss below that expected if no sedative or anesthetic had been used This led us to examine the effect of a barbiturate on the fluid loss from a burned surface and to compare the fluid loss in such a case with that which occurred when no sedation or anesthetic was administered.

Mechanism of Production of the Observed Effects. How a barbiturate effects curtailment of fluid loss from damaged capillaries is not certain Under the circumstances of this experiment, the effect is not accomplished through a general blood pressure lowering effect, for arterial blood pressures were carefully followed (rabbits) and found to be the same in control and test animals This was to be expected since relatively small doses of barbiturates were used Although the mode of action of the barbiturates in reducing the fluid loss is not clear, two chief possibilities may be mentioned.

(*1*) *The barbiturate may have reduced the flow of capillary filtrate by causing capillary contraction* with reduction of the extent of the filtering surface. Support for this view can be found in the observations of Bennett, Bassett and Beecher (1943) that sudden increase in depth of barbiturate anesthesia is associated with an often demonstrable increase in peripheral resistance to blood flow. Moreover, the pale, cool skin customarily found in man under barbiturate anesthesia may be the result of peripheral constriction of blood vessels This explanation appears to be a more likely one than the following:

(*2*) *The barbiturate may have lowered the capillary pressure in the tissues studied.* Evidence for such a view can be found not only in the effect of the agent in diminishing the fluid loss from the burned surface but also in its effect in reducing the capillary dilatation and congestion of the duodenal mucosa If we assume that the barbiturate does lower capillary pressure, then it lowers the filtration pressure. While there is no direct evidence that this is the case, such an assumption can form a working hypothesis and it also offers a tentative explanation for the following puzzling data: Bollman, Svirbelly and Mann (1938) reported that the plasma volume of dogs increased 9% under sodium amytal anesthesia. A similar increase occurred in the absence of the spleen. In agreement with this, Hamlin and Gregersen (1939) found that pentobarbital sodium causes a 10% increase in plasma volume in normal cats. Suppose at the moment the barbiturate was injected that a condition of near equilibrium existed between the filtration pressure inside the capillary and the pressure of the filtrate outside. With lowering of the capillary filtration pressure it is possible that fluid would return from the tissues to the blood stream, and that an actual increase in plasma volume would result. As mentioned, this has been observed. Support for the view that the contractile mechanism of the blood vessels is concerned in the production of these barbiturate effects is found in three[2] observations of Hamlin and Gregersen (loc. cit.) that the plasma diluting effect of the barbiturates does not appear in their sympathectomized cats. (In this case they report increases of only 2% or less.)

The interference with metabolic processes known to be caused by barbiturates may result in a decreased formation of synthetic water. While it hardly seems *likely that this could be of importance here, it might possibly be a contributing* factor. Available data do not warrant at this time an attempt to give a complete discussion of the possible mechanisms involved. In any such discussion the effects of barbiturates on the permeability of membranes would have to be considered. From the hematocrit data presented in table 1, it appears unlikely that changes in blood viscosity could account for the phenomenon observed.

In the present study the data obtained are concerned with fluid loss, well known to be of importance in the development of burn shock, rather than, at the moment, with the development of shock itself. In a subsequent study it will be interesting to observe the effect of barbiturates on plasma volume in the presence of large weeping burns and to observe the relationship of plasma volume to fluid loss as shock develops. It is of importance to know where the fluid is that is not lost from the burned surface in these animals which have received barbiturates, and in this connection we have not been able to find fluid accumulation outside of the circulatory system. It is reasonable to suppose that even if this "saved" fluid has not remained within the blood stream (as all available evidence suggests is the case) it is not lost to the body and can be reabsorbed into the blood stream.

Clinical Implications of the Observed Effects. Even if man lost no more fluid in terms of percentage of his body weight than rabbits in response to a burn (certainly not the case), the difference in fluid loss effected by the barbiturate might well be critical.[3] In the case of a 75 kgm. man, if he loses fluid as chickens do in a 4 hour period following a burn of one third of his body surface, he will lose about 3.4% of his body weight through the burned area, that is about 2550

[2] A fourth experiment had to be ruled out, since regrowth of splanchnic connections may have occurred.

[3] See Cannon's discussion (1923) of the importance of "critical further bleeding"—widely recognized by all who deal with hemorrhage either in man or in experimental animals.

cc whereas if he had received barbiturates to the point of sedation this loss would have been 2 6% of his body weight, about 1930 cc , a saving of some 600 cc of fluid nearly as rich in protein as plasma, equivalent to the plasma from a liter of normal blood Since this 600 cc "plasma' loss comes on top of a 1900 cc loss in the subjects without barbiturate its importance is evident[3]

Certainly any claims as to the value of barbiturates in the therapy of burns are not yet called for, althought enough experimentel data are at hand to suggest a clinical test of the matter The following considerations strengthen this point of view The use of barbiturates to curtail the fluid loss from a burned surface does not interfere with or replace any standard burn therapy The procedure is particularly well adapted to field use and use under all circumstances in the interval before plasma replacement therapy can be started or where the plasma supply is limited The procedure in tending to dry up the skin is advantageous for certain forms of burn surface therapy The barbiturates have a desirable sedative and somewhat analgesic action Small doses are effective Morphine has of course great importance in the control of pain in patients with extensive burns, and is used in large dosage for this purpose There was the possibility that morphine might also curtail fluid loss There would be little use in employing barbiturates in these cases if morphine also curtailed fluid loss, accordingly, morphine was tested in this regard Under the conditions of these experiments morphine has no effect on the fluid loss from a burned surface

SUMMARY AND CONCLUSIONS

This study demonstrates that when tissues are damaged by heat that the resulting loss of fluid and protein from the burned surface can be reduced significantly by a barbiturate Pentobarbital sodium (nembutal) in sedative dose was used to demonstrate this effect in chickens (preliminary observations) and finally in rabbits In the latter animals two types of tissue were burned (a) skin and (b) small intestine with its mesentery

In the experiments reported here, it was important to eliminate or circumvent two primary difficulties (a) Pain during the experimental procedures overcome by the use of decortication (chickens), local anesthesia (abdominal wall in rabbits), and low spinal cord transection (skin burns in rabbits) (b) Skin structure of animals dissimilar to that of man This apparently is responsible for the smaller degree of weeping of the burned surface in animals than is true in the case of man To study the effect of barbiturates when the effect of overlying layers of skin was not a factor burns of the small intestine and mesentery were carried out

The barbiturate was administered following the burn The quantity of fluid loss from the burned surface was determined by weight difference The respiratory water loss was determined and found to be less in the rabbits with barbiturate than in the controls accordingly corrections were made for this

On examining the animals no extravascular accumulation of fluid was found in the skin burn experiments It seems probable that the fluid remaining within the animal as a result of the barbiturate remained within the vascular system

While a final statement on this question waits on plasma volume and other body fluid volume studies, direct evidence supporting this view was found in less capillary and lymphatic congestion demonstrated by microscopic pathological examination in the animals with barbiturate as compared with the controls.

The mechanism of action of the barbiturate effect is not clear. The suggestion was made that it might be accounted for by a reduction (a) in capillary filtering surface or (b) in capillary pressure. Evidence was cited that the vascular contractile mechanism may be concerned. The effects reported were not due to differences in systemic arterial pressure between the barbiturate and control groups of rabbits.

A much drier skin surface is obtained, following a burn, when barbiturates are used than when they are not. Much less capillary congestion of the duodenal mucosa was observed in the animals with skin burn when barbiturates were used than when they were not.

Calculations were made suggesting that if a man lost no more fluid than animals following a burn that the fluid and protein saved by the use of barbiturates might be critical in circumstances where plasma replacement therapy was not available or was delayed. Reasons are offered as to why the barbiturate effect described here merits a clinical study in the treatment of burns.

Finally, studies were carried out with morphine since it is already widely employed in the treatment of burns. Morphine has no curtailing effect on fluid loss as does the barbiturate.

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SODIUM SUCCINATE AS AN ANTIDOTE FOR BARBITURATE POISONING AND IN THE CONTROL OF THE DURATION OF BARBITURATE ANESTHESIA

(Including Its Successful Use in a Case of Barbiturate Poisoning in a Human)

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The currently accepted treatment of barbiturate poisoning is the stimulation of the central nervous system by more or less specific drugs (1). The best results have been obtained with picrotoxin (2) which stimulates the midbrain and the medulla, and particularly the depressed respiratory centre (3). However, picrotoxin is in itself a dangerous drug, the margin of safety between its effective and lethal dosages being rather small (4).

The work of Quastel and Wheatley (5) upon the mechanism by which barbiturate depresses the respiration of the brain tissue, suggested to us a new and safer treatment for barbiturate poisoning. These authors reported that barbiturates decreased the Qo_2 of brain tissue *in vitro*, by inhibiting the oxidation of glucose, lactate and pyruvate. The oxidation of succinate was not affected. It occurred to us that by supplying sufficient of the latter substrate, one might adequately maintain the metabolism of the brain of a poisoned animal until the barbiturate had been destroyed or excreted. We found that succinate was, indeed, an effective antidote for barbiturate poisoning. We also found that, following the administration of anesthetic doses of barbiturate, succinate could be used to control the duration of the anesthesia.

The recovery of rats from nembutal poisoning. Male rats of the Long-Evans strain, weighing 200 ± 20 grams, were used throughout. Food was removed from their cages 16 hours prior to the experiments but water was allowed *ad libitum*. Into each animal the M.L.D. established by Carmichael (6), namely, 8.5 mgm. of sodium pentobarbital (Nembutal) per 100 grams body weight was injected intraperitoneally as an 8.5% aqueous solution

We are aware of the differences of opinion which exist as to the best criteria for the duration of anesthesia. The end point is not sharply defined by any standard. We measured the duration of anesthesia from the moment the animal collapsed and no longer responded to noxious stimuli, to the time when it spontaneously assumed a normal upright position.

Our first attempts to use sodium succinate as an antidote were to inject varying amounts of an aqueous solution subcutaneously some minutes after the ani-

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mals had collapsed. The results were so irregular as to suggest that, in many animals, the dose of anesthetic used was sufficiently toxic to render the circulation too slow for effective absorption of the succinate. We therefore turned to intravenous injection of the succinate into the external jugular vein, using 25 mgm. per body weight, dissolved in 1 cc. of distilled water. Of 20 control animals injected with 1 cc. saline, 13 died, while only 4 of the 20 succinate-treated rats succumbed.

While the results of the intravenous injection of succinate appeared to be significant, this procedure in so small an animal rendered the work laborious and time-consuming, and unexpected difficulties interfered with the maintenance of constant time intervals. To obviate these difficulties but at the same time assure the absorption of the succinate, we finally settled on the intramuscular injection of the antidote (100 mgm. per 100 grams body weight in 1 cc. of water) 15 minutes prior to the administration of the anesthetic. Table 1 shows the results of this treatment in 40 rats compared with 40 control rats. It may be seen that 34 (85%) of the succinate-treated animals survived while only 18 (45%) of the control animals recovered.

Each number in the columns of table 1 represents a different rat. It also gives the time in minutes which elapsed from the collapse of the animal after nembutal injection, to its death or to its recovery as the case might be. The numbers in each column have been arranged in order of magnitude to show the extreme variability of the time factor in this work, and the futility of calculating average times for death or recovery. However, the median times for the groups (indicated by the horizontal line across each column) suggest that the succinate-treated animals took a longer time to die or recovered more quickly than the corresponding control animals.

THE CONTROL OF THE DURATION OF BARBITURATE ANESTHESIA IN RATS. The influence of succinate on the duration of barbiturate action was much more clearly shown when, instead of toxic doses, the usual anesthetic doses of nembutal were used. Hence the latter were also used to test the possible beneficial effects of glucose and of carbohydrate intermediates other than succinate. Similarly, anesthetic rather than toxic doses of sodium amytal were used in the further testing of succinate.

Figure 1 graphically demonstrates the influence of different amounts of sodium succinate on the effect of an anesthetic dose of nembutal. 2.5 mgm. of nembutal per 100 grams body weight, in 2.5% aqueous solution, injected intraperitoneally, was used throughout. The type and size of animal and the conditions prior to experiment were as described in the previous section. The amounts of sodium succinate used varied from 0.05–50 mgm. per 100 grams body weight. The particular dose of succinate used was dissolved in 1 cc. of distilled water and was injected intramuscularly, 5–7 minutes after the administration of the nembutal.

The heavy horizontal bar across each block in figure 1 represents the median duration of anesthesia for a particular group of animals. The times for individual rats are plotted along each block to show the number of animals and the scatter of values from which the median was derived. Although the great time-variability seen in the poisoning experiments persisted, the results in the

TABLE 1

Incidence and times of death and of recovery respectively, of rats given 8 5 mg nembutal per 100 gm body weight intraperitoneally

The succinate treated rats received 100 mg sodium succinate per 100 gm body weight, 15 minutes prior to the injection of the barbiturate The horizontal lines across the columns indicate the median time for each group

CONTROL		SUCCINATE TREATED	
Died	Recovered	Died	Recovered
minutes	*minutes*	*minutes*	*minutes*
15	68	34	66
21	180	37	86
22	180	90	90
23	186	—	95
28	187	90	104
28	190	94	118
35	202	130	127
42	202		128
48	206		147
52	—		153
66	209		157
—	215		160
68	229		166
86	246		170
93	246		178
131	248		179
152	253		182
163	256		—
182	276		191
215			209
216			228
222			231
225			231
			238
			238
			242
			260
			260
			261
			270
			271
			281
			281
			288
			311
Total rats 22	18	6	34
Percentage survived	45		85

present experiments are unequivocal As little as 5 mgm of sodium succinate per 100 grams body weight reduced the median duration of anesthesia to half the control value, while 37 5 mgm per 100 grams body weight reduced it to one third

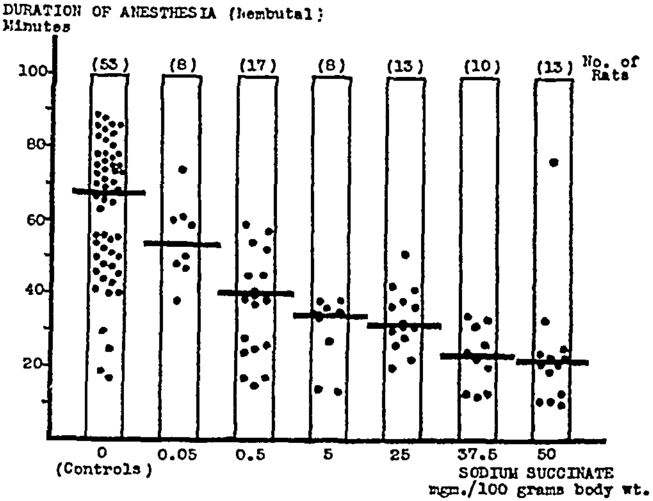

FIG. 1. THE INFLUENCE OF DIFFERENT AMOUNTS OF SODIUM SUCCINATE ON THE DURATION OF NEMBUTAL ANESTHESIA

The dots in each block represent times for individual rats. The horizontal bar across each block indicates the median time for the group. The number of rats in each group is indicated in parenthesis above the block.

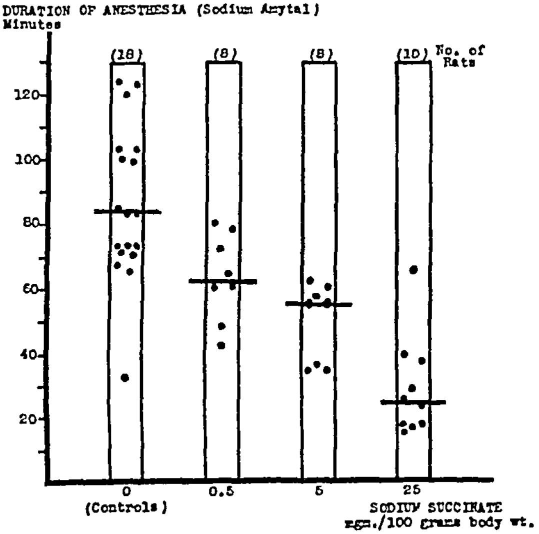

FIG. 2. THE INFLUENCE OF DIFFERENT AMOUNTS OF SODIUM SUCCINATE ON THE DURATION OF SODIUM AMYTAL ANESTHESIA

Conventions as for figure 1

of the control. In spite of the relatively small number of animals in each of the treated groups, the graded effects obtained with graded dosages are very convincing. The statistical significance of the results with each dose of succinate is indicated by the value of Fisher's Student "*t*" (by which a *t* value of 2.0 or above is significant). For the 0.05 mgm. dose, *t* = 1.2; 0.5 mgm., *t* = 5.4; 5 mgm., *t* = 4.9; 25 mgm., *t* = 5.6; 37.5 mgm., *t* = 6.6; 50 mgm., *t* = 7.9. Because of the variance in the control group, a further statistical analysis of the relation of dosage to time was made, using the "*F*" test. It was found that *F* = 12.8 (3.3 or above is significant).

Figure 2 summarizes another series of experiments similar in all respects to the above except that sodium amytal, (5 mgm. per 100 grams body weight) was used

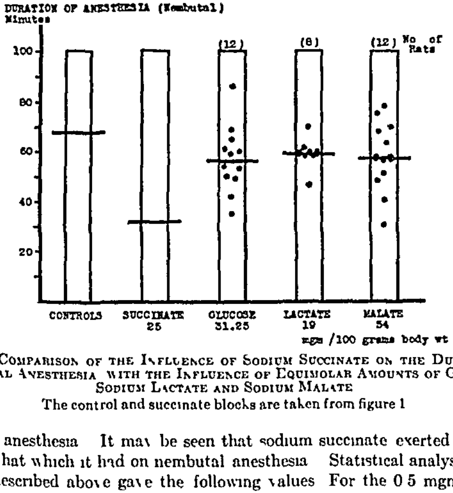

FIG. 3. A COMPARISON OF THE INFLUENCE OF SODIUM SUCCINATE ON THE DURATION OF NEMBUTAL ANESTHESIA WITH THE INFLUENCE OF EQUIMOLAR AMOUNTS OF GLUCOSE, SODIUM LACTATE AND SODIUM MALATE

The control and succinate blocks are taken from figure 1.

to induce anesthesia. It may be seen that sodium succinate exerted a similar effect to that which it had on nembutal anesthesia. Statistical analysis by the method described above gave the following values: For the 0.5 mgm. dose, *t* = 2.5; 5 mgm., *t* = 4.4; 25 mgm., *t* = 6.9. Quantitative comparisons with the nembutal results are not justified in view of the smaller number of animals used.

It may be mentioned in passing that a number of preliminary tests of sodium succinate on the anesthesia induced by tribromethanol or Avertin (as representative of non barbiturates) yielded no effects whatsoever.

Figure 3 compares the influence of sodium succinate on nembutal anesthesia with the influence of equimolar amounts of glucose, sodium lactate and sodium malate. Although the doubtful reduction in the median duration of anesthesia produced by the latter substances is rather consistent (and suggests further trials with larger dosages), there is no comparison between these effects and the influence of an equimolar amount of succinate. The *t* value for the succinate was 5.6, while that for equimolar amounts of glucose, lactate, and malate was 1.3, 1.2 and 1.0 respectively.

SAFETY OF SODIUM SUCCINATE. Since succinate is a normally occurring intermediate substance in metabolism, the possibility of toxic side reactions from its use could be discounted in advance. But, for the sake of completeness, some normal rats were subjected to extremely large doses. 600 mgm. of sodium succinate per 100 grams body weight were injected intramuscularly in 8 rats. This was 6 times the largest amount we had used to counteract toxic doses of barbiturate and 12 times the largest amount we had used to shorten the duration of anesthesia. It was equivalent to a dose of 420 grams to a 70 kgm. man, and was the largest amount it was practical to give intramuscularly in the rat from the standpoint of volume and concentration. It was necessary to administer the total amount in 6 divided doses; 200 mgm. in 1 cc. distilled water into each hind leg, three times at 20 minutes intervals.

The animals were sacrificed 24 hours later, at which time they appeared perfectly normal. Post-mortem examination revealed no gross abnormality in any tissue or organ. Histological examination of sections of the kidneys and livers stained with hematoxylin-eosin, did not disclose any pathological changes in the kidneys, but did present an appearance of the hepatic tissue which it was difficult to interpret. However, when sections of the liver were stained for glycogen with the Best carmine stain, they showed unusually rich deposits of glycogen sufficient to account for the findings with the other stain. Chemical analysis of the livers supported the microscopic findings, and revealed amounts of glycogen up to 7%. It may be concluded that there is no evidence that even extremely large amounts of succinate exert any harmful influence.

SPECIES DIFFERENCE IN THE SUCCINATE EFFECT, AND THE SUCCESSFUL USE OF SUCCINATE IN A CASE OF BARBITURATE POISONING OF A HUMAN. The effectiveness in one species of animal of a procedure such as has been described is, of course no guarantee of its therapeutic possibilities in another species. For example, in a small number of tests on normal dogs anesthetized with nembutal, we were unable to demonstrate any striking effects of succinate in doses comparable to those used in rats. When, however, we tested succinate on abdominally eviscerated dogs, it was at least as effective as in normal rats. It seems probable that the liver of the dog is capable of disposing of large quantities of succinate, and that therapeutically effective doses of succinate for the normal dog would have to be much larger than for the rat. In view of this it is of value to report that, in a case of barbiturate poisoning of a human which happened to become available to us, the use of sodium succinate appeared to be of benefit.

The individual concerned was a 63 year old woman, weighing 110 pounds, who had taken an estimated 3.5 grams of barbiturates in the forms of Seconal and Allonal. The drugs were taken in the evening; vigorous treatment was begun the following morning. The treatment included large amounts of picrotoxin, totalling 1083 mgm. by the morning of the third day. However, at this time the patient was still deeply comatose and the case appeared rather hopeless. It was then felt justified to discontinue orthodox treatment and to try sodium succinate as a last resort. During the next 5 hours, 22 grams of sodium succinate was injected intravenously, as a 10% aqueous solution. By the end of this procedure the patient was awake and responded to visual and auditory stimuli. She went on to complete recovery without further treatment.

It is not implied that this single human case proves anything. But it does suggest that the results obtained on the rat are applicable to man, and that further work with human material is indicated.

SUMMARY AND CONCLUSIONS

1. It has been shown that sodium succinate is a safe and effective antidote against toxic doses of nembutal in rats For best results it should be given intravenously after the barbiturate has been administered; if given prophylactically before the barbiturate, it may be injected intramuscularly or intravenously.

2. Sodium succinate has also been shown to shorten the duration of both nembutal and sodium amytal anesthesia in rats, the degree of shortening depending upon the dose of succinate given. For this purpose, the succinate may be administered intramuscularly or intravenously after the anesthesia has been induced.

3. Glucose, sodium lactate and sodium malate were also tested, and did not produce effects comparable to succinate.

4. A case of barbiturate poisoning in a human is described, in which the use of sodium succinate appeared to be of great benefit

5. Further work on human subjects should be done to establish: (a) the possibility of rendering routine intravenous barbiturate anesthesia a safe and practical procedure by the prophylactic use of sodium succinate (b) the possibility of terminating intravenous barbiturate anesthesia at will, by adequate treatment with sodium succinate after the induction of anesthesia (c) the value of sodium succinate in barbiturate poisoning.

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THE CHRONIC TOXICITY OF THE SULFONAMIDES FOR THE GROWING RAT AS INFLUENCED BY THE TYPE OF DIET, THE ADDITION OF FECES TO THE DIET, AND APPETITE

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The chronic toxicity of the sulfonamides for the rat has been studied by a number of workers, who have shown that sulfanilamide (1), sulfapyridine (2), sulfathiazole (2, 3), sulfaguanidine (4, 5), and sulfasuxidine (6) inhibit growth. In addition to such well known effects as stones in the bladder and damage to the kidney, and as particularly associated with sulfanilamide, anemia and methemoglobinemia, other lesions have been described including an eosinophilic interstitial myocarditis (7), thyroid enlargement and hyperemia (8), hyaline sclerosis and calcification of the blood vessels and voluntary muscles, agranulocytosis, and dermatitis (9, 10, 11).

The toxicity of the sulfonamides is reduced by a number of substances. In the case of rats on a low protein diet (7%), raising the protein to 30% or adding 0.5% methionine and 0.1% cystine reduced the mortality due to sulfanilamide (12). This result is at least partly explained by the finding that increased protein intake is associated with increased urinary secretion, and therefore with lower blood levels of the drug (13); the addition of the amino acids also lowered the concentration of drug in the blood (12).

This type of physiological explanation, however, does not seem to cover the other known cases of antagonism. Thus for rats on 18% casein diets (5), small amounts of liver extract or of *p*-aminobenzoic acid, even when injected subcutaneously, antagonized sulfaguanidine (0.5% in diet). Sulfasuxidine (1% in diet) was also antagonized by liver extract, but not by *p*-aminobenzoic acid (6); it was not toxic when incorporated in a commercial chow which presumably contained meat. Biotin (10) markedly increased the span of life of rats on a purified diet containing 18% of casein and 1% of sulfaguanidine, though it was unable to prevent the development of various blood vessel lesions. It also cured the dermatitis which developed when such animals were saved from death by the administration of liver extract over long periods of time. Also, it has been shown (14) that yeast extract or dried rat feces can increase the rate of growth of rats partially inhibited by sulfaguanidine.

It has been suggested (5, 14) in explanation of the foregoing that sulfaguanidine, and also sulfasuxidine, act primarily upon the intestinal flora, thus preventing the synthesis of vitamins essential to the rat, and, indeed, it was shown that a hypoprothrombinemia was induced which could be cured by the administration of vitamin K Also, *p* aminobenzoic acid (15) and methionine (16, 17) are sulfonamide antagonists in bacteria However, the fact that *p* aminobenzoic acid does not antagonize sulfasuxidine argues that other factors must be at work, such as the direct action of the drugs upon the tissues of the animals

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[1] Now on active duty, M. C., U. S. A.

[2] We wish to thank the Rockefeller Foundation for a grant in aid of this work.

With the foregoing as a general background, we wish to present our data pertaining to the relative chronic toxicity of the various sulfonamides when these are exhibited in the rat at blood levels commonly employed in medical practice Such data are not in the literature, partly because most workers have tested but a single drug at a time, partly because the blood level of the drug in question was not determined during the course of the experiment

From what has been said, it is obvious that any determination of the relative chronic toxicity is necessarily true only for the diet actually employed in its determination We have therefore tested a number of drugs incorporated in two different types of diet, the protein content remaining approximately constant Both the food consumption and the gain in weight of young rats have been followed because these seem to be the most sensitive criteria available Such data may be of interest from the pediatric point of view Furthermore, the usual toxic manifestations of the sulfonamides observed clinically are of the mild rather than of the seriously acute type

METHODS White rats of the Vanderbilt strain, about 4 5 weeks old, weighing 50–70 grams were kept in individual wire cages Water was provided *ad libitum* The food consumption was determined daily, when necessary, by weighing the earthenware food cups (or special metal cups) placed in each cage The food consisted of either Purina dog chow (Checkers) ground to a powder, or a purified diet In either case the sulfonamides were administered by mixing them intimately with the food Both diets supported growth well According to the manufacturer, Purina chow contains protein 22 5% fat 5 5%, carbohydrate 50 25% fiber 3 75%, and ash 7 7% The purified diet contained Labco casein 25%, Wesson oil 8% sucrose 60%, and salts 7% To each kilo of purified diet there were added the following supplements choline chloride 500 mgm calcium pantothenate 40 mgm riboflavin 5 mgm thiamin chloride 2 5 mgm , pyridoxin 2 5 mgm vitamin K 1 25 mgm In addition each rat on the purified diet received by mouth three times a week one drop of wheat germ oil (Squibb) and one drop of Mead's blended oil

In the paired feeding experiments the animals were arranged in pairs The experimental animal received the sulfonamide containing diet *ad libitum* the food consumption for each day being noted The food cup of the control animal received each day, of food containing no sulfonamide, only as many grams as the experimental animal had consumed the day before

Blood sulfonamide levels were determined from tail blood drawn between 1 and 4 p m , using the method of Bratton and Marshall (18) The level was taken as the difference between the experimental and the controls Hemoglobin determinations were done according to Evelyn and Malloy (19) By total hemoglobin is meant the sum of the hemoglobin, oxyhemoglobin, methemoglobin, and sulfhemoglobin The total hemoglobin was not reduced by more than 20% in any case

EXPERIMENTAL RESULTS *Results with commercial chow* The rate of growth as a function of time was approximately linear for about 50 days in the case of the males fed commercial chow, with or without sulfonamide In the case of the females, the relationship was somewhat curvilinear We estimated the rate of growth in either case by determining the number of days required for the males to gain 100 grams, and the females to gain 70 grams. In table 1 the *rate of growth* is given as a percentage of the control rate. Two types of control were employed, one fed ad libitum, the other paired-fed. The controls were always run simultaneously with the experimentals.

As seen in figure 1, 1% sulfanilamide is slightly less toxic than 1% sulfathiazole, although its total level in the blood of 16.8 mg.% (determined on the 43 day) was twice that of the sulfathiazole. These and additional data are presented in table 1 (groups 1, 2, 5, 6, and 9). The table also shows that sulfathiazole is not inhibitory until the dietary level is above 0.3% and the blood level above 3 mg.% (groups 3 and 4). Since the food consumption of the inhibited animals was less than that of the controls by as much as 25% (table 1), a paired-feeding experiment was done to determine whether food consumption per se was the limiting factor. This was found to be the case, as seen in figure 2, and in table 1, groups 7 and 8.

TABLE 1

In almost all cases, the control animals gained from 3 to 4.5 grams per day, depending upon their sex and age. The growth rate of the experimental animals is stated as a percentage of the controls: 100 × (days for male controls to gain 100 grams, or females to gain 70)/(days for experimentals to gain the same amount).

GROUP AND DIET	SEX AND NUMBER	DRUG	BLOOD LEVEL		GROWTH RATE		FOOD CONSUMPTION
			Free	*Total*	% ad lib. control	% paired-fed control	
			mgm.%	*mgm.%*			*% control*
1	♂ (7)	1% SA	12.8	16.8	64		80†
2	♀ (7)	1% SA	13.7		62		89
3	♂ (5)	0.1% ST		1.5	100		
4	♂ (5)	0.3% ST		3.4	94		
5	♂ (7)	1% ST	6.4	8.2	53		73
6	♀ (7)	1% ST	7.5	7.7	56		76
7	♂ (8)	1.0% ST	5.7	6.6		103	
8	♀ (8)	1.0% ST	5.8	6.7		104	
9	♂ (5)	1% ST			68*		79
10	♂ (8)	1% SG	2.9	3.7	140		113
11	♀ (8)	1% SG	2.9	3.0	132		120
12	♂ (5)	1% SG			100*		95
13	♂ (5)	0 25% SD	8.0	8.0	113		
14	♂ (5)	0.8% SD	23.0	22.5	61	100	

* Based on the time to gain 50 grams.

† That a 20% reduction in food consumption is associated with 40% reduction in growth rate is explained by the fact that the animal must eat most of his food to avoid losing weight.

To determine the possible influence of *p*-aminobenzoic acid, 4 males were fed chow containing 1% sulfathiazole and compared with 4 fed chow containing 1% sulfathiazole plus 1% *p*-aminobenzoic acid. It was found that the mean time to gain 100 grams for the *p*-aminobenzoic acid group was 42 days, for the sulfathiazole controls 44.5, a difference of 6%, which was not significant.

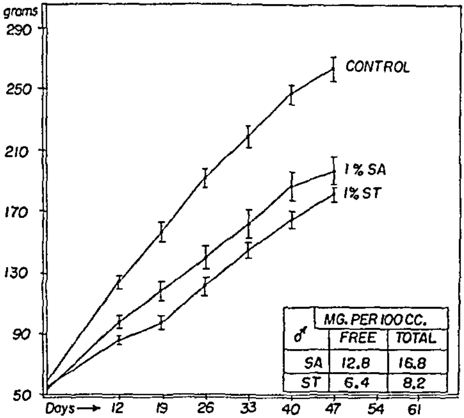

FIG. 1. GAIN IN WEIGHT OF MALE RATS FED COMMERCIAL CHOW CONTAINING 1% OF SULFANILAMIDE OR OF SULFATHIAZOLE

The blood level of drug is indicated in the table

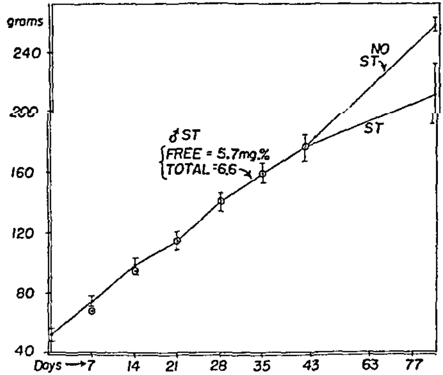

FIG. 2. GAIN IN WEIGHT OF MALE RATS FED COMMERCIAL CHOW CONTAINING 1% OF SULFATHIAZOLE (DOTS WITH STANDARD ERRORS)

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than the other three animals which continued to receive the drug

In the case of sulfaguanidine at a level of 1% in the diet, resulting in a total blood level of 3–4 mg.%, an increase in the rate of growth accompanied by increased food consumption was noted in rats from one pooling of litters (groups 10 and 11), while in those from another pooling there was no effect (group 12).

Sulfadiazine fed at a dietary level of 0.25% resulted in a blood level of 8 mgm.% and a slightly increased rate of growth. At a dietary level of 0.8% and a blood level of 22.5 mgm.%, the growth rate was 61% of the *ad libitum* control, and 100% of the paired-fed control. Here again the food consumption was the limiting factor in growth.

We may summarize these results as follows.

1. The inhibition of growth results from decreased food consumption, and not from a decrease in the efficiency of utilization of ingested food. *p*-Aminobenzoic acid does not antagonize the inhibition. *Consequently, the inhibitory actions of the sulfonamides under these conditions may involve nothing more than an inhibition of the centers controlling appetite.*

2. At a given dietary level, sulfadiazine produces the highest blood levels, followed by sulfanilamide, sulfathiazole, and sulfaguanidine. In general, less that 25% of the total blood level is acetylated.

3. On the basis of total blood levels, sulfaguanidine at 3–4 mgm.% ($1.4–1.9 \times 10^{-4}$M) and sulfadiazine at 8 mgm.% (3.2×10^{-4}M) are non-toxic; sulfathiazole at 6.5–8 mgm.% ($2.6–3.2 \times 10^{-4}$M) is slightly more toxic than sulfanilamide at 17 mgm.% (1×10^{-3}M) and sulfadiazine at 23 mgm.% (9.2×10^{-4}M). A similar difference between sulfathiazole and sulfadiazine was reported by Feinstone *et al.* (3).

Results with purified diet. It will be recalled that the inhibitions observed with the commercial chow were relatively constant, particularly in the males, during a period of 45 days (fig. 1). On the purified diet, however, three phases may be observed (fig. 3): *induction*, during which the inhibition first appears and grows; *fixation*, during which the inhibition is constant and the animal may even lose weight; and *escape*, during which the inhibition decreases markedly. To evaluate the relative toxicity of the drugs, each phase must be taken into consideration: induction, sulfaguanidine acts most rapidly; fixation, all drugs brought the weekly gain in weight to less than 5 grams; escape, both the low sulfathiazole and the low sulfaguanidine escaped. All things considered, on the basis of dietary level, sulfathiazole is the least toxic, while sulfadiazine is the most toxic. On the basis of the blood level, sulfaguanidine is the most toxic. Furthermore, sulfaguanidine is more toxic than sulfathiazole on the basis of either blood level or dietary level. On the 49th day, two of three animals on 1% sulfaguanidine had red encrustations on their noses, and one was quite dirty. All of the animals on 1% sulfathiazole seemed to be in better condition and were slowly gaining weight. This order of toxicity is different from that obtained with commercial chow and shows that the relative toxicity depends on the diet.

Antagonism by feces. The recent report by Light *et al.* (14) that the addition of feces to the diet increased the rate of growth of rats on a ration containing sulfaguanidine led us to the following experiment. Each group of six animals which had been on a drug diet for 23–29 days was split into two subgroups of three One subgroup continued on the drug diet as before, members of the other received the drug diet plus about 200 mgm dry weight of feces, which were mixed in the food cup each day The feces were collected each morning from the papers beneath the wire floors of the cages housing animals fed drug free purified ration, and were prepared by pulverizing after drying at 90°C for 24 hours The addition of the feces to the diet began a day or so following the first sulfonamide blood level analysis, indicated by the arrows in figure 3

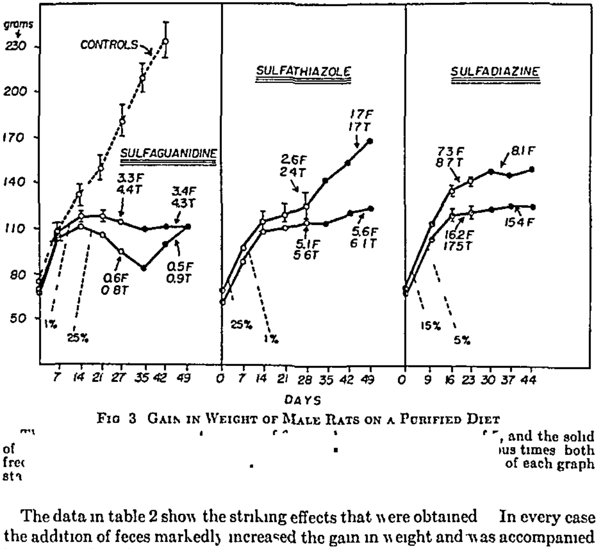

FIG 3 GAIN IN WEIGHT OF MALE RATS ON A PURIFIED DIET

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The data in table 2 show the striking effects that were obtained In every case the addition of feces markedly increased the gain in weight and was accompanied by increased food consumption, and this effect was noticed before the end of the first week The full effect, however, was not seen in those animals whose growth had been stopped completely, or almost so, until the second and third weeks Thus in the case of 0 15% SD, the animals receiving feces gained 27 5 grams during the second week, whereas those not receiving feces lost 1 3 grams During this period the controls on drug free diet were gaining 25–30 grams a week This establishes beyond question that the feces actually antagonize the drugs Although Light *et al* (14) concluded this to be the case, their data only suggested this conclusion They found that animals on a drug free purified ration gained

83 grams in 4 weeks, those receiving sulfaguanidine gained 75% as much, and those receiving drug and feces gained 124% as much. Such data equally well could be interpreted to mean that the rate of growth was limited by the diet itself, and the addition of feces provided a useful supplement.

Table 2 also proves that the increase in growth due to the addition of feces was not the result of a lowered blood level of sulfonamide, a factor not previously considered. In no group was the mean blood level lower in the animals receiving feces, and in about half the groups it was somewhat higher. Although the number of animals in each group was small, the complete agreement makes the result significant.

TABLE 2

The effect of the addition of feces to the diet

Feces were added to the ST and SG diets starting on the thirtieth day, and to the SD diet starting on the twenty-fourth day. The daily dose was about 200 mg., dry weight.

DRUG IN DIET	FECES	NO. OF ANIMALS	DAYS	WEIGHT[*] GAINED	DAYS	WEIGHT GAINED	SULFONAMIDE IN BLOOD	
							Free	Total
				grams		*grams*	*mgm. %*	*mgm. %*
1% ST	–	3	29–35	−3 ±1.52	43–49	+2 ±5.5	5.6	6.1
1% ST	+	3	29–35	+4.3 ±1.8	43–49	+18.7 ±2.7	7.5	8.7
0.25% ST	–	3	29–35	+18 ±3.8	43–49	+14.3 ±2.9	1.7	1.7
0.25% ST	+	3	29–35	+31 ±3	43–49	+31 ±8.2	2.2	2.1
0.5% SD	–	3	24–30	+2.3 ±2.33	31–37	+3.7 ± .35	15.4	
0.5% SD	+	2	24–30	+14.5 ±2	31–37	+30 ±5	15.4	
0.15% SD	–	3	24–30	+5.7 ±1.2	31–37	−1.3 ±3.6	7.8	
0.15% SD	+	2	24–30	+12 ±2	31–37	+27.5 ±1.42	8.1	
1% SG	–	3	28–35	−4.7 ±0 88	43–49	+.7 ±1.8	3.4	4.3
1% SG	+	3	28–35	+7 ±2.1	43–49	+22.7 ±4.9	4.7	5.6
0.25% SG	–	3	28–35	−10.3 ±2.4	43–49	+14 ±6 25	0.5	0.9
0.25% SG	+	3	28–35	+8.3 ±5.3	43–49	+17 ±5.2	0.5	1.0

[*] Controls gained 30 grams during 29–35 days and ate 87 grams.

The question of whether these results prove the sulfonamides are toxic chiefly by virtue of their action upon the intestinal flora still remains, in our opinion, an open one. It has not yet been established in the rat that normally the intestinal flora release significant amounts of dietary essentials (other than K and probably biotin) which are absorbed. It should also be recalled that Welch (6) failed to antagonize sulfasuxidine with *p*-aminobenzoic acid, and we failed using sulfathiazole. Furthermore, the relative order of toxicity of the sulfonamides for the rat does not correspond to their effectiveness in reducing the coliform count of the stools in mice (the only species for which it has been determined). Using adult mice fed Wayne Fox Chow containing 1% sulfonamide, White (20) found sulfadiazine to be somewhat more active than sulfathiazole, which was twice as active as sulfaguanidine. Sulfanilamide was without effect. These results check with neither our Purina chow nor purified ration series.

SUMMARY

1. Young rats were fed diets containing the various sulfonamides, and their rate of growth, food consumption and blood drug-level were determined.

2. *When the diet was a commercial chow*, the following data were obtained. Sulfaguanidine at a total blood level of 3–4 mgm.% (1.4–1.9 × 10^{-4}M) and sulfadiazine at 8 mgm.% (3.2 × 10^{-4}M) did not inhibit. Sulfathiazole at 6.5–8 mgm.% (2.6–3.2 × 10^{-4}M) inhibited the rate of growth by 45%, sulfanilamide at 17 mgm.% (1 × 10^{-3}M) by 37%, and sulfadiazine at 23 mgm.% (9.2 × 10^{-4}M) by 40%. It was proved by paired-feeding experiments that the sulfathiazole and sulfadiazine inhibitions were due to the decrease in food consumption. *p*-Aminobenzoic acid failed to antagonize sulfathiazole

3. *When the diet was a purified ration*, the source of protein being casein, all of the drugs were much more toxic. The blood levels listed above under 2 were able to bring the weekly gain in weight to less than 5 grams (compared to 25–30 grams for the controls) after about 2 weeks on the diet.

4. Sulfathiazole at 2 mgm.% (total blood level) and sulfaguanidine at 1 mgm.% were unable to maintain the inhibitions they had induced, and after a variable period these animals, which had stopped, or almost stopped growing achieved half the normal rate of growth.

5. On the basis of dietary level, sulfadiazine is the most toxic, and sulfathiazole the least. On the basis of the blood level, sulfaguanidine is the most toxic. Hence the relative order of toxicity of sulfathiazole, sulfaguanidine, and sulfadiazine changes with the diet, as shown by comparing these data with those under 2.

6. The daily addition to the diet of about 200 mgm. of dry feces from drug free animals produces a striking increase in the rate of growth of animals completely inhibited by the sulfonamides. This effect is not due to a decrease in the blood level of sulfonamide.

We wish to thank the Lederle Laboratories for a gift of sulfadiazine and sulfaguanidine.

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STUDIES ON OINTMENTS[1]

I. Penetration of Various Ointment Bases

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What happens to the ointment when rubbed into the skin? Does it act as a food so to speak, for the skin? Is it really absorbed? Is it metabolized? These questions have engaged the interest of many investigators from time to time. However, absorption was often confused with penetration and the conclusions drawn by the different workers were contradictory, mainly due to the use of different methods and choice of different test objects.

Oppenheim (1) stated that there is no local influence on the skin the sense of nutritive ointments. Jolles (2) wrote that locally applied fats, according to some authors, are absorbed by the blood or lymph stream. Miyazaki (3) thought, that the entire skin absorbs and that adeps suillus, lanolin, olive oil and petrolatum are absorbed. Moser and Wernli (4) found relatively large amounts of fatty acids in the urine after percutaneous application, which suggested absorption of fats. Latzel and Steyskal (5) went further and attempted to provide patients in whom the oral feeding was difficult, with the necessary calories through the skin in form of fat, protein and carbohydrate inunction. Their claimed results were much disputed. Winternitz and Naumann (6) found that the absorption of fats is below 1/1500 of the applied amount, which makes the nutrition through the skin very problematic. Bernard and Strauch (7) were of the same opinion and found that hydrocarbons are entirely unabsorbable. It was pointed out by these workers that the disappearance of the ointment from the skin surface does not necessarily mean absorption, for it may penetrate into the skin by a process which is for the most part emulsification and from here only very minute amounts may be absorbed; however the largest part is wiped off. This view is most generally accepted.

The dermatologist is not so much interested in the absorption of a base, which is of little practical importance to him, but in the penetration of it. It is therefore not surprising that the first report on this subject came from a dermatologist. Working in Unna's laboratories, Sutton (8) in the year 1906 reported that he found almost no penetration of lanolin and petrolatum, but good penetration of goose grease after four hours in the skin of rabbits. Unna and Frey (9) in 1929 made experiments on guinea pigs. They incorporated india ink into various bases, rubbed these into the skin and examined the penetration in microscopic sections. They found no penetration of petrolatum but some penetration of water-in-oil (w/o) emulsions. As india ink is not soluble in the bases used, their experiments were interesting but not conclusive. A much better method for the study of penetration was recommended by Bauschinger (10) in 1938 who incorporated into the bases to be examined a fat- or water-soluble dye depending upon the kind of base. The penetration of the dye is a measure for the ointment penetration, as the oil-soluble dye forms a unit with the fatty medium and the water soluble, with the watery medium. This method or modifications of it have subsequently been widely used.

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[1] From a thesis submitted to the Graduate Faculty of the University of Minnesota in partial fulfillment for the degree of Master of Science in Dermatology and Syphilology, in March 1942

Eller and Wolff (11) in 1939 applied olive oil, *cocoa butter, cold liver oil and* hydrous wool fat and liquid petrolatum and petrolatum to the backs of six mature albino rabbits, which were clipped prior to the application of the test material. Biopsies were performed after two, four and six hours and the frozen sections were stained with Sudan IV. They found that fats permeate the skin along the hairshafts and into the oil gland ducts and that liquid fats permeate the skin more rapidly that solid ones. It was further shown that animal fats had the greatest depth of penetration, with vegetable fats next and mineral fats last. Most of the fats showed optimum penetration between four and six hours after application. Duemling (12), who also used rabbits, employed a similar method in determining the penetration of ointments to which wetting agents were added. He found a remarkable penetration of the fatty substance almost to the base of the hair follicle, even after 15 minutes. After one hour the fat had completely disappeared from the superficial portions of the subcutaneous areas but was still present in the deepest portion of the specimen. Harry (13) used for his experiments human post-mortem material, the skin of rats and checked some of his findings on the skin of a human leg prior to amputation.

Almost all these experiments were done on animal skin and do not necessarily apply to the living human skin. Most of the experiments were done on rabbits, which are not very satisfactory for this purpose, on account of the large sebaceous glands in the skin and the slender margin of its keratinous protective layer. Moreoever one must bear in mind, that shaving, depilation or even clipping may have changed the normal impermeability of the skin.

It seemed therefore worthwhile to reinvestigate the penetration of ointment bases using only human skin.

EXPERIMENTAL METHODS. All experiments were done on human skin only. Every experiment was repeated at least four times. In order to determine whether the penetration of the base varies with the age of the individual, the studies were done on three groups of individuals: Group A, whose ages ranged from 5 to 15 years; Group B, whose ages were between 20 and 50 years; Group C, whose ages were between 55 and 70 years. Half the individuals were males and half females. The following substances were tested: (1) Lard, (2) Cod liver oil, (3) Olive oil, (4) Petrolatum, (5) Vaseline (Chesebrough), (6) Lanolin, (7) Water-in-oil emulsions (w/o), including: (a) Petrolatum and lanolin to equal parts. (b) "Aquaphor," a base consisting of 6 per cent of a group of esters of cholesterol in an aliphatic hydrocarbon base.[2] (c) Petrolatum and 5% cetyl alcohol.

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[2] Furnished by Duke Laboratories, Inc., Stamford, Conn.

(d)	Mannide monooleate	10%[3]
	ceresin wax	10%
	petrolatum light	15%
	mineral oil	45%
	lanolin	20%

(e) "Hydrosorb," a base consisting of oleic acid esters and amide of diethanolamine, oleic acid and white petrolatum [4]

(8) Oil in water emulsions (o/w), including (a) Rose water ointment U S P

(b)	Lecithin	5 0
	petrolatum	45 0
	water	50 0
(c)	Stearyl alcohol	10 0
	water	10 0
	mineral oil	10 0
	petrolatum	70 0
(d)	Liquid petrolatum	56 0
	peanut oil	3 0
	triethanolamine	2 0
	stearic acid	4 0
	cetyl alcohol	3 0
	water	72 0

Sudan IV was added to the oils, fats, hydrocarbons and emulsions where it was desired to follow the oily phase, in quantities enough to give the ointment a deep red color A water soluble dye, such as methylene blue or trypan blue, was added where it was desired to follow the aqueous phase In every case the dye was thoroughly mixed with the ointment

Technique One gram of each ointment was applied by means of a glass spatula to the skin of the anterior aspect of the thigh, forearms, back or abdomen Only such massage as was necessary to spread the ointment with the spatula over the skin area, was used The areas (3 x 3 inches) were not covered and precautions were taken, especially in children, that the ointments were not rubbed off or massaged in by the individual Biopsies were performed after 15 and 30 minutes, 1, 2, 4, 6, 12 and even after 24 hours in some cases Block anesthesia (1% procaine) was used in every case and the skin area as such was not touched at all After the biopsies were performed by means of a punch, the wounds were thoroughly rinsed with antiseptics and dressed Only once was there even a slight infection of the biopsy wound

The technique used for the determination of the fat penetration is similar to the one used by Eller and Wolff (11) and Duemling (12) with the exception that the dyes were added to the bases and that every fifth to tenth section was stained with Nile blue sulfate

Preliminary experiments were done in which symmetric untreated areas were used as controls This was necessary to become familiar with the variation of the normal fat content in and around the different structures of the skin and to study the possible objection to the technique, namely that the stained areas observed were actually the ointment bases and did not represent the penetration of the dye itself It must be admitted that the penetration of the incorporated dye caused some confusion at the beginning, especially in the sebaceous glands, but it was soon found that the color of the particles of the base was considerably darker than the areas stained by the dye, which penetrated from the base Counter staining with Nile blue sulfate was very helpful, since the particles of the ointment base represented themselves as little globules of reddish orange color, while the normal fat was stained red

No fat was ever found along the hairshaft, hairbulb and around the sweat or sebaceous penetration of water-in-oil (w/o) emulsions. As india ink is not soluble in the bases used, their experiments were interesting but not conclusive. A much better method for the study of penetration was recommended by Bauschinger (10) in 1938 who incorporated into the bases to be examined a fat- or water-soluble dye depending upon the kind of base. The penetration of the dye is a measure for the ointment penetration, as the oil-soluble dye forms a unit with the fatty medium and the water soluble, with the watery medium. This method or modifications of it have subsequently been widely used.

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[3] Furnished by Wm S Merrell Comp , Cincinnati, Ohio

[4] Furnished by Abbott Laboratories, North Chicago, Ill

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[2] Furnished by Duke Laboratories, Inc., Stamford, Conn.

dividuals. The speed of penetration decreases with the age. For this I have no good explanation except that the tonus and the metabolism, which also decreases with the age, may be partly responsible. There is no penetration through the *stratum corneum*. The ointment were never found deeper than the upper looser layer of the *stratum corneum* and this is certainly only due to mechanical effect of spreading the ointment on the skin. This confirms the observations made by Eller and Wolff (11), Harry (13) and Miescher (14) and is definitely contrary to Unna's conception. He advocated the theory that fats, especially when they are emulsified, will penetrate through the skin. Unna (15) believed that the cells of the epidermis contain esters of oleic acid and argued that the fatty ointment bases will emulsify with these esters and penetrate from cell layer to cell layer, through the entire epidermis until it reaches the corium. However, he was never able to present convincing proof for his theory, which was based on the assumption that the dark particles in the epidermis, especially in the basal cell layer, which he saw after staining with osmic acid, were oleic acid. Patzelt (16), however, was able to prove that Unna's assumption was incorrect. He showed that Unna's findings with osmic acid could be obtained even after the skin was completely defatted and that staining with Sudan, which is specific for esters of fatty acids, even using a special technique, gave completely negative results. It was further pointed out by Patzelt that staining fat with osmium depends only on chemical reduction and therefore cannot be considered as a specific test, as long as other important criteria are lacking.

Therefore one cannot speak of a fat content in the *stratum corneum*, at least not in the sense that substances with the physical properties of fats occur as an integral part in the cells of the *stratum corneum* and around the sweat pores as deposits of products from the sebum and sweat secretion.

The bases penetrate only inside along the hairshaft and into the sebaceous glands. I was unable to determine the further path of the penetration, namely from the sebaceous glands to the subcutis, where some of the different ointment bases eventually appear in the form of irregular conglomerated foreign masses.

The relative intensity of the penetration of the different tested substances listed in the order from the best to the worst penetration, as revealed by this study is as follows: base No. 8 d (liquid petrolatum-peanut oil-triethanolamine-stearic acid-cetyl alcohol-water), "Aquaphor" (Duke) and "Hydrosorb" (Abbott) showed for all practical purposes the same penetration, then come lard, cod liver oil, stearyl alcohol-mineral oil-water-petrolatum, lanolin, base No. 7 d (Merrell), lecithin ointment, petrolatum and lanolin to equal parts, petrolatum plus five per cent cetyl alcohol, olive oil, rose water ointment, vaseline (Chesebrough) and petrolatum.

Relation of the hydrogen ion concentration of the skin and the pH value of bases. The discussion of ointment bases as such would be incomplete without briefly considering their pH and their relation to the hydrogen ion concentration of the skin.

We know since the work of Unna's pupil Heuss (17), that the skin surface is acid. Sharlit and Scheer (18) found the pH of the surface of the skin, around 5.5.

Their findings were confirmed by Memmesheimer (19), Schade and Marchionini (20) and others. Marchionini (21) showed later, that the skin in the axilla, in genital and rectal regions and under the breasts in females, is alkaline. The pH values of the skin according to these investigators are between 3 and 5 for the skin surface or stratum corneum, between 5.5 and 6.4 for the stratum granulosum, between 6.7 and 6.9 for the stratum spinosum, between 7 and 7.4 for the stratum basale and between 7.4 and 7.5 for the cutis. It was further pointed out by Marchionini (22) that the acid reaction of the skin surface is the most important mechanism against bacterial growth. Alkaline reacting creams or ointments should therefore only be used in exceptional cases on the healthy skin otherwise, the most important bulwark against infection will be destroyed and the living condition for pathogenic organism be improved.

TABLE 2

BASES	pH VALUE
Lard (pooled)	6.8
Petrolatum (pooled)	8
Vaseline (Chesebrough)	7
Lanolin (pooled)	6.5
Petrolatum-Lanolin to equal parts	7.5
Aquaphor (Duke)	7
Petrolatum plus 5% cetyl alcohol	7.5
Hydrosorb (Abbott)	6 8
Stearyl alcohol-water-mineral oil-petrol.	7 8
Rose water ointment USP. (pooled)	6 5
Liquid petrolatum-peanut oil-triethanolamine stearic acid-cetyl alcohol-water	6 9

Table 2 shows the pH values of the eleven bases which were used in the penetration study.[5]

SUMMARY

1. Experimental studies on the penetration of the following substances: lard, cod liver oil, olive oil, petrolatum, vaseline (Chesebrough), lanolin, petrolatum and lanolin to equal parts, "Aquaphor" (Duke), petrolatum plus five per cent cetyl alcohol, a base consisting of mannide monooleate-ceresin wax-petrolatum-mineral oil-lanolin, "Hydrosorb" (Abbott), rose water ointment USP., lecithin ointment, a base consisting of stearyl alcohol-mineral oil-water-petrolatum and a base consisting of liquid petrolatum-peanut oil-triethanolamine-stearic acid-cetyl alcohol and water, were reported.

The relative intensity of the penetration into the normal human skin of the different test substances listed in the order from the best to the worst penetration, as revealed by this study is as follows: the base consisting of: liquid petrolatum-peanut oil steric acid triethanolamine acetyl alcohol and water, "Hydrosorb" (Abbott), "Aquaphor" (Duke), lard, cold liver oil, stearyl alcohol mineral oil water petrolatum, lanolin, a base consisting of mannide monooleate ceresin wax petrolatum mineral oil lanolin, lecithin ointment, petrolatum and lanolin to equal parts, petrolatum plus five per cent cetyl alcohol, olive oil, rose water ointment, vaseline (Chesebrough), and finally petrolatum as such

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[5] I wish here to thank Dr George R Hazel from the Abbott Lab, North-Chicago, Ill, for his kind help in determining the pH values.

2 The relation of the hydrogen ion concentration of the skin and the pH values of bases was discussed and the pH values of 11 bases used in this study are listed

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THE BIOLOGICAL ASSAY OF POSTERIOR PITUITARY SOLUTION

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The purpose of this investigation was to develop a relatively simple procedure for the biological assay of the oxytocic activity of posterior pituitary solutions which would permit an objective estimate of the reliability of each determination. The study was deemed advisable because of the widely recognized fact that the method using the guinea pig uterus possesses many disadvantages. These disadvantages have been set forth by Morrell, Allmark, and Bachinski (1), who have evolved a new method which also employs the guinea pig uterus, but which is based on a quantal response. Their method is so designed that the error of each individual test may be computed. In the present investigation the problem was attacked by applying modern experimental design and analysis to the chicken blood pressure test for oxytocic activity reported by Coon (2) and later studied by Smith (3).

Coon's method is based on the fact, first reported by Paton and Watson (4), that the intravenous injection of posterior pituitary solution into the bird elicits an immediate, marked, and transitory fall in blood pressure. His procedure consists of injecting standard and unknown solutions alternately until doses of the two which produce equal falls of pressure are determined. Although the test possesses the advantages of relative simplicity and economy, two objections may be noted. First, the method leaves to the discretion of the assayist the decision as to which responses may be considered equal. Falls in pressure which are *equal* are unusual, and the interpretation is further complicated by tachyphylaxis. The second objection is the difficulty of computing the experimental error of each assay.

EXPERIMENTAL. Experiments calculated to elucidate the dose-response curve were carried out and the assay designed according to the precepts of Bliss and Marks (5, 6).

Three curves relating dose and response are shown in figure 1. Analysis of the data upon which these and other similar curves are based showed that the response is linear, when plotted against the logarithm of the dose. The various curves do not differ significantly in slope, but the position of the curve varies with the sensitivity of the bird.

The assay procedure as finally evolved is as follows. A white leghorn rooster is selected, anesthetized, set up for intravenous injection and the blood pressure recorded according to the directions of Coon (2). One cc. of U.S.P. standard posterior pituitary solution is diluted to 10 cc. with 0.9% NaCl solution, and the solution to be tested is diluted with the same diluent in such a manner that it is expected to be equal in potency to the diluted standard solution. The dose of diluted standard solution which will produce a fall in blood pressure of approximately 20 mm Hg is determined, usually 0 10 cc ± 0 05 cc This dose designated as the low dose of the standard (S_1) and twice this amount is designated as the high dose of the standard (S_2) An amount of diluted unknown solution equal in volume to the low dose of the standard is the low dose of the unknown (U_1), and an amount equal in volume to the high dose of the standard is the high dose of the unknown (U_2) These four doses are each administered four times, making a total of sixteen injections, with a ten minute interval between doses The injection schedule is set up by assigning the various doses at random to a four by four latin square (5) When rapid development of tachyphylaxis makes it apparent that the response to the low dose may soon

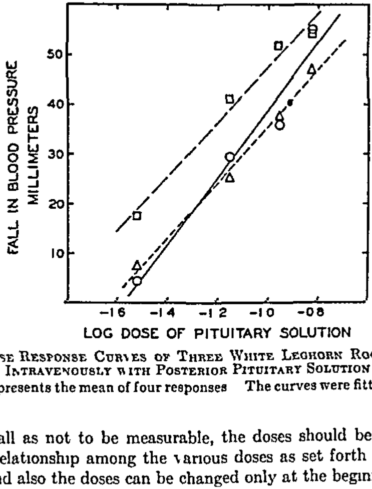

FIG 1 THE DOSE RESPONSE CURVES OF THREE WHITE LEGHORN ROOSTERS INJECTED INTRAVENOUSLY WITH POSTERIOR PITUITARY SOLUTION

Each point represents the mean of four responses The curves were fitted by the method of least squares

become so small as not to be measurable, the doses should be increased In doing so the relationship among the various doses as set forth above must be maintained, and also the doses can be changed only at the beginning of a group of four injections, i e , beginning with injections number 5, 9, or 13 If a clot forms in the cannula, and this complication may be expected not infrequently, the clot should be washed out, the last dose preceding the wash out repeated and the response ignored Then the regular schedule of injections is resumed Each response as indicated by the fall in blood pressure is measured in millimeters Protocols of a typical assay together with details of calculation are shown in tables 1 to 4

In table 3 the sum of the products is obtained by multiplying the totals, Yp, obtained from table 2, by the appropriate factorial coefficients and summing

TABLE 1

Protocol of assay

7-16-42. Bird no. 22. 1740 grams. 200 mgm./kgm. Na Phenobarbital I.M.

INJECTION NUMBER	TIME	PREPARATION AND DOSE	DOSE	FALL IN PRESSURE
			cc.	*mm.*
1	12:45	S_1	0.10	18
2	12:55	S_2	0.20	34
3	1:05	U_1	0.10	25
4	1:15	U_2	0.20	36
5	1:25	S_2	0.20	35
6	1:35	U_2	0.20	32
7	1:45	S_1	0.10	12
8	1:55	U_1	0.10	18
9	2:05	U_2	0.24	36
10	2:15	U_1	0.12	17
11	2:25	S_2	0.24	30
12	2:35	S_1	0.12	11
13	2:45	U_1	0.15	19
14	2:55	S_1	0.15	16
15	3:05	U_2	0.30	40
16	3:15	S_2	0.30	28

TABLE 2

Calculation of data—A

SETS	FALLS IN BLOOD PRESSURE (Y) (MM.)				Row totals
	S_1	S_2	U_1	U_2	
1st	18	34	25	36	113
2nd	12	35	18	32	97
3rd	11	30	17	36	94
4th	16	28	19	40	103
Totals = Y_P . .	57	127	79	144	407 = SY

TABLE 3

Calculation of Data—B

Factorial analysis of data from table 2

TREATMENT EFFECT	FACTORIAL COEFFICIENTS (x) FOR DOSE				DIVISOR† $NS(x^2)$	SUM OF PRODUCTS $S(xY_P)$	VARIANCE $\frac{S^2(xY_P)}{NS(x^2)}$	STANDARD DEVIATION $\sqrt{\text{VARIANCE}}$
	S_1	S_2	U_1	U_2				
Samples.	−1	−1	+1	+1	16	39	95.06 = D^2	9.75 = D‡
Slope	−1	+1	−1	+1	16	135	1139.06 = B^2	33.75 = B
Parallelism	+1	−1	−1	+1	16	−5	1.56	
Totals Y_P*.	57	127	79	144				

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* From Table 2.

† S = sum of; N = number of times each dose is given.

‡ The standard deviation is always given the same sign as the corresponding sum of products.

the products For example, the sum of the products for the difference between samples is calculated as

$$S(xYp) = (-1)(57) + (-1)(127) + (+1)(79) + (+1)(144) = 39$$

The variance for the same treatment effect is calculated by squaring the sum of the products and dividing by the corresponding divisor, thus

$$\text{Variance} = \frac{S^2(xYp)}{NS(x^2)} = \frac{39^2}{16} = 95.06$$

The standard deviation is then calculated as

$$\text{Standard deviation} = \sqrt{\text{variance}} = \sqrt{95.06} = 9.75$$

The corresponding values for the other treatment effects, slope and parallelism are obtained similarly

After the values in Table 3 are calculated, the potency is computed as

$$M = \frac{.3010\, D}{B},$$

where M is the logarithm of the potency, 0.3010 is the logarithm of the ratio of the high dose to the low dose, and D and B are taken directly from Table 3 For this example the potency is calculated as

$$M = \frac{(0.3010)(9.75)}{33.75} = .0870$$

$$\begin{matrix}\text{Potency} \\ \text{in} \\ \text{per cent}\end{matrix} = 100\ (\text{antilog } M) = 100\ (\text{antilog } 0.0870) = 122$$

The standard error of M is calculated as

$$s_M = \frac{s\,0.3010\,\sqrt{B^2 + D^2}}{B^2},$$

where s is the root mean square for experimental error taken directly from the analysis of variance which follows (table 4) and the other terms have the same significance as in the equation for determining M Finally, the average standard error of the relative potency is calculated by means of Cochran's formula

$$\text{s.e. of relative potency in per cent} = 2.303\ s_M\ (\text{antilog } M)\ 100$$

Substituting the values of the present example

$$s_M = \frac{(3.047)(0.3010)\sqrt{1234.12}}{1139.06} = 0.0283, \text{ and}$$

$$\begin{matrix}\text{s.e.} \\ \text{in per cent}\end{matrix} = (2.303)(0.0283)(1.22)(100) = 8.0$$

The estimated potency of the unknown is therefore 122% ± 8.0% of the standard, a figure which agrees with the true potency, 117.6%

The analysis of variance is a mathematical tool developed by Fisher (7) which is used here (table 4) for isolating the variation in response due to experimental error from that due to known factors such as the differences between doses and tachyphylaxis. The variation due to each of these sources is calculated as the sum of the squares from the data presented in table 2. The first step is the computation of the correction factor, $\frac{(SY)^2}{16}$, which is used in obtaining the sums of the squares. This factor is obtained by squaring the sum of the sixteen original observations (falls in blood pressure) and dividing by the number of observations. For the present example this is

$$\frac{407^2}{16} = 10{,}353.06.$$

TABLE 4

Calculation of data—C

Isolation of variation due to experimental error by the analysis of variance.

VARIATION DUE TO	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Differences between doses	3	1235.69	
Differences between sets	3	52.69	
Experimental error	9	83.56	9.284*
Total	15	1371.94	

* Root mean square for experimental error $(s) = \sqrt{9.284} = 3.047$

The sum of the squares for the differences between doses is calculated from the column totals of table 2 as

$$\frac{57^2 + 127^2 + 79^2 + 144^2}{4} - \frac{407^2}{16} = 1235.69,$$

for the differences between sets, due principally to tachyphylaxis, from the row totals in table 2, as

$$\frac{113^2 + 97^2 + 94^2 + 103^2}{4} - \frac{407^2}{16} = 52.69,$$

and for the total sum of the squares from the sixteen original observations as

$$18^2 + 12^2 + 11^2 + \cdots + 40^2 - \frac{407^2}{16} = 1371.94.$$

The sum of the squares for the experimental error then follows as the remainder after the sums of the squares for the differences between doses and for the differences between sets have been subtracted from the total sum of the squares. For the present example this is calculated as

$$1371.94 - 1235.69 - 52.69 = 83.56.$$

The degrees of freedom associated with each entry in the analysis of variance, table 4, are obtained by subtracting one from the number of the squares entering a given sum Thus in the case of "doses' and "sets" the degrees of freedom are 4 − 1 = 3, and for the total sum of the squares 16 − 1 = 15 The degrees of freedom for experimental error are obtained by subtracting those for "doses" and "sets' from the total as 15 − (3 + 3) = 9 For a sixteen dose assay of this type the degrees of freedom for each entry remain constant

The mean square for the experimental error is obtained by dividing the sum of the squares by the degrees of freedom, and the root mean square for experimental error is the statistics, which was used for calculating the standard error of M

TABLE 5

Assays of solutions of known potency

ASSAY NUMBER	TRUE POTENCY	POTENCY FOUND	STANDARD ERROR	ACTUAL ERROR AS PERCENT OF TRUE POTENCY
	per cent	*per cent*	*per cent*	
1	80	79	5 2	1 2
2	81	73	6 0	9 9
3	111	108	6 5	2 7
4	72	78	5 2	8 3
5	94	90	6 0	4 3
6	111	110	5 8	1 0
7	75	71	7 7	5 3
8	110	130	27 2	18 2
9	118	122	7 9	3 4
10	67	79	12 2	17 9
11	70	70	5 9	0
12	110	122	11 7	10 9
13	135	126	12 4	6 7
Mean				6 9

Thirteen solutions of known potency have been assayed and calculated in the same manner as the preceding example These known solutions were prepared by dilution of the U S P standard posterior pituitary solution, and in each case the assayist was ignorant of the true potency until the results of the assay had been calculated The data obtained in these experiments are set forth in table 5

DISCUSSION In the method of calculation presented above certain potential sources of variation are ignored These are the influence of the height of the blood pressure immediately preceding a given response and the influence of the size of the preceding dose Analysis of the data obtained in several assays by means of partial regression coefficients showed that the minor fluctuations in pressure normally encountered and the effect of the preceding dose can be neglected without appreciably diminishing the accuracy of assays Variation due to tachyphylaxis is largely taken care of by the experimental design and method of analysis. Further refinements in the latter accomplish only a slight reduction in the standard error.

Burn (8) has written in his standard work on biological assay that

> "Methods are good if they are accurate, rapid, and simple and bad if they are inaccurate, slow, and need skill. That accuracy is necessary all are agreed. That speed in obtaining a result is important is . . . well known to those engaged in estimations on behalf of commercial firms. Still fewer people recognize that methods are good in proportion as the technique is simple."

The method reported fulfills these requirements. The accuracy is attested by the data set forth in table 5. The average deviation of potencies found from the true potencies is only 6.9%.

The technique of the assay is simple, and anyone familiar with the ordinary manometric method usually used for measuring blood pressure can master it. Although the mathematics underlying the method of computation are intricate, their understanding is not necessary to the assayist, who has only to substitute in the appropriate formulae and use arithmetic to calculate the results of an assay.

An occasional bird must be discarded because of very low blood pressure, tachyphylaxis which develops with excessive rapidity, numerous extrasystoles, or relative insensitivity, but on the whole the method is economical in both time and funds. A single determination can be carried out and calculated in approximately four hours. White leghorn roosters are more readily available and less expensive per acceptable assay than guinea pigs suitable for the usual uterine test.

SUMMARY AND CONCLUSIONS

1. A method is presented for the biological assay of the oxytocic activity of posterior pituitary solutions.
2. The mean error of thirteen assays of solutions of known potency was 6.9%.
3. The reliability of each assay can be determined independently.
4. The method is simple and economical.

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THE CHEMICAL NATURE OF COMPOUNDS WHICH INHIBIT THE FUNCTION OF THE THYROID GLAND[1]

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Inhibition of function in an endocrine gland by the administration of chemical compounds is a new field of investigation in endocrinology. Pharmacological studies have not heretofore been concerned with drugs exhibiting this type of activity, but recent work indicates that such compounds may be uncovered and in the case of the thyroid gland certain agents have been found to possess the specific property of inhibiting thyroid hormone production.

The administration to rats of sulfaguanidine (1), phenylthiourea (2) or allylthiourea (3) has been found to result in a pronounced enlargement and hyperplasia of the thyroid gland, an action that is common to a number of sulfonamides and thiourea derivatives (4, 5). This effect is not modified by increasing the iodide intake but is abolished if thyroid hormone be given concurrently (4, 5). The thyroid hyperplasia is accompanied by a decreased food intake, a lessened rate of body growth and development, and a lowered basal oxygen consumption. These compounds do not influence the thyroid in the absence of the hypophysis and have no inhibitory action upon the calorigenic activity of artificially administered thyroid hormone (5). Thus it appears that the primary action of compounds of the type mentioned above is concerned with the inhibition of thyroid hormone production.

In view of the therapeutic importance (6) of drugs which have the specific property of decreasing the formation and liberation of the hormone of the thyroid gland, a study has been made on a number of compounds in order to determine the chemical groupings responsible for this type of activity. It was anticipated that this information would permit the development of more highly active agents of low toxicity.

Méthods. In order to determine the relative effectiveness of different substances in inhibiting thyroid hormone production, estimates were made of the degree of compensatory thyroid hyperplasia that followed their administration in various dosages The substances to be tested were administered to normal young female rats, 21 to 30 days of age, for periods of 10 days The readily soluble compounds and those easily dissolved by the addition of acid or alkali were dissolved in the drinking water. The relatively insoluble substances and the larger doses of the more soluble ones were admixed with the food. The volume of water drunk and the weight of food consumed were measured and the animals were weighed daily. The amount of the drug which each animal received daily was estimated from the quantity consumed by the group over the 10 day period and this amount was expressed on a body weight basis using the average body weight of the group of animals over the period of the test. In each test 2 to 4 animals were used for each dosage level, and from 6 to 18 such groups were run simultaneously. The animals were killed with illuminating gas and the thyroid region dissected out without touching the gland and without removing it from its attachments to the trachea and larynx. The degree of thyroid effect was graded preliminarily as negative or as one plus to four plus upon the gross appearance of hyperplasia as indicated by increased size and hyperemia. The glands were fixed in formalin and paraffin sections were stained with hematoxylin and eosin; the final grading of the degree of hyperplasia was made from the microscopic appearance. The criteria used in forming an estimate of the degree of hyperplasia are shown in table 1.

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[1] This work was done under the auspices of the University Committee on Pharmacotherapy.

Both the gross and the microscopic inspections were done without knowledge of the dosage or the nature of the compound being tested and in nearly every instance the two criteria agreed closely. Whenever there was doubt as to whether the glands were slightly hyperplastic or normal the test was considered to be negative. When a compound was found to be active at a given dosage, the substance was retested at higher and lower levels in order to approximate the minimal effective dose and to determine the amount required to induce a maximal response. Certain active compounds were toxic and could be tested only at doses which induced minimal or moderate thyroid hyperplasia. Substances which proved to be inactive were retested at higher levels, and in most instances the dose was increased until toxic levels were reached.

TABLE 1

Grading of the degree of hyperplasia from the microscopic appearance of the thyroid glands

ENLARGEMENT AND PROLIFERATION OF ACINAR CELLS	*COLLOID LOSS*	ESTIMATE OF HYPERPLASIA
Slight or none	Minimal	–
Distinct	Moderate	+
Moderate	Over half of total	++
Marked	Nearly complete	+++
Maximal	Complete	++++

Results. *Thiourea and related compounds.* The most active compounds tested belong to a group of substances possessing a thiourea grouping. In table 2 are shown the results obtained with 32 compounds which may be considered to be derivatives of thiourea, as all of these substances have in common the thioureylene radical: —NH·CS·NH—. Thiourea itself is quite active, a maximal effect being produced by a dose of 13 mgm. per 100 grams of body weight per day. This substance, although disagreeable to the taste in human beings, was well tolerated by rats and the daily oral administration of 2 grams to adult rats was not lethal over a period of 10 days. Alkyl and aryl derivatives of thiourea were not more active than the parent compound with the exception of *sym*-diethyl thiourea, and certain derivatives were less active. Most of the derivatives were more toxic than thiourea, particularly the amino, acetyl, allyl, guanyl, phenyl, *o*-tolyl, and di-*o*-tolyl derivatives. The di-*n*-butyl, diphenyl and di-*p*-tolyl derivatives were of very low potency, possibly because of their low solubility in water.

Substituted *2*-thiohydantoins were quite active, the *1*-acetyl and *5* benzal derivatives being slightly more active than thiourea but all of these compounds

TABLE 2

Derivatives of thiourea

COMPOUND	FORMULA	METHOD OF ADMINISTRATION[1]	DAILY BODY WEIGHT GAIN[2]	AVERAGE DOSE	THYROID RESPONSE
		%	grams	mg / 100 gm / day	
Thiourea	NH_2CSNH_2	0 005 water	3 7	1 2	−
		0 01 water	2 4	2 1	+
		0 02 water	3 3	3 3	++
		0 10 water	1 8	13 1	++++
		1 00 water	1 1	117 0	++++
Methylthiourea	$NH_2CSNHCH_3$	0 01 water	3 0	2 1	+
		0 02 water	3 7	4 6	++
		0 10 water	2 1	44 4	++++
Thiosemicarbazide	$NH_2CSNHNH_2$	0 05 water	−2 2	5 1	+
Acetylthiourea	$NH_2CSNHCOCH_3$	0 02 water	2 0	4 0	+
		0 10 water	0 3	13 0	+++
Allylthiourea	$NH_2CSNHC_3H_5$	0 01 water	3 3	2 1	+
		0 02 water	2 7	4 2	++
		0 10 water	0 6	4 9	+++
Guanylthiourea	$NH_2CSNHC(NH)NH_2$	0 02 water	2 5	3 4	−
		0 05 water	0 6	5 1	−
Sym Diethyl thiourea	$C_2H_5NHCSNHC_2H_5$	0 002 water	3 8	0 6	+
		0 005 water	3 7	1 3	++
		0 01 water	2 2	2 5	+++
		0 10 water	0 5	20 2	++++
Sym Di n butyl thiourea	$C_4H_9NHCSNHC_4H_9$	1 00 food	−0 3	185 0	++
Phenylthiourea	$NH_2CSNHC_6H_5$	0 01 water	2 7	1 7	−
		0 05 water	−0 2	2 5	−
		0 10 water	died		
o Tolylthiourea	$NH_2CSNHC_6H_4CH_3$	0 10 water	−1 1	1 7	−
		1 00 food	died		
Phenylthiohydantoic acid	$C_6H_5NHCSNHCH_2COOH$	0 10 water	2 6	18 3	−
		0 50 food	−1 0	60 0	+
		1 00 food	−0 1	115 0	−
Sym Diphenyl thiourea	$C_6H_5NHCSNHC_6H_5$	5 00 food	−0 8	530 0	++
Sym Di p tolyl thiourea	$CH_3C_6H_4NHCSNHC_6H_4CH_3$	1 00 food	3 2	134 0	−
Sym Di *o* tolyl-thiourea	$CH_3C_6H_4NHCSNHC_6H_4CH_3$	0 50 food	2 2	66 7	++
		2 00 food	died	83 6	−
Dithiobiuret	$NH_2CSNHCSNH_2$	0 002 water	died	0 3	−
		0 05 water	died	0 9	−

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[1] The percentages are grams of solid or cc of liquid in *100 c c of solution or in 100 grams* of food

[2] The average daily body weight gain of 7 groups totaling 32 control animals was 2 5 to 3 4, mean 3 1 ± 0 13 grams

TABLE 2—*Concluded*

COMPOUND	FORMULA	METHOD OF ADMINISTRATION[1]	DAILY BODY WEIGHT GAIN[2]	AVERAGE DOSE	THYROID RESPONSE
		%	*grams*	*mg/100 gm./day*	
Methylisothiourea sulfate	$\mathrm{(NHC(SCH_3)NH_2)_2H_2SO_4}$	0.10 water	1.2	11.1	++
		0.50 water	0.1	25.2	+
1-Acetyl-*2*-thiohydantoin	$\underbrace{\mathrm{N(COCH_3)CSNHCOC}}\mathrm{H_2}$	0.01 water	3.1	3.0	+
		0.10 water	0.6	9.7	+++
5-Benzal-*2*-thiohydantoin	$\underbrace{\mathrm{NHCSNHCOC}}\mathrm{(CHC_6H_5)}$	0.005 water	3.7	1.3	+
		0.01 water	3.9	2.4	++
		01.0 water	1.5	16.5	++++
5-Furfural-*2*-thiohydantoin	$\underbrace{\mathrm{NHCSNHCOC}}\mathrm{(CHC_4H_3O)}$	0.10 water	1.9	20.6	++
1-Benzoyl-*2*-thiohydantoin	$\underbrace{\mathrm{N(COC_6H_5)CSNHCOC}}\mathrm{H_2}$	0.10 water	2.5	26.2	–
		1.00 food	*died*	*147.0*	–
2-Aminothiazole	$\underbrace{\mathrm{SC(NH_2)NCHCH}}$	0.10 water	0.7	10.3	++
		0.50 water	–0.6	148.2	+++
Thioammeline	$\underbrace{\mathit{NHCSNHC(NH)NHC}}\mathit{(NH)}$	0.02 water	3.1	6.1	–
		1.00 food	1.8	123.0	++
Monothiocyanuric acid	$\underbrace{\mathrm{NHCSNHCONHCO}}$	0.01 water	2.8	3.2	–
		0.10 water	1.3	30.0	–
		0.50 water	died		–
Dithioammelide	$\underbrace{\mathrm{NHCSNHC(NH)NHCS}}$	0.01 water	3.5	2.2	–
		0.10 water	4.3	25.0	–
		1.00 food	1.6	118.2	++
2-Thiouracil	$\underbrace{\mathrm{NHCSNHCOCHCH}}$	0.002 water	4.3	0.6	+
		0.005 water	3.4	1.5	+
		0.01 water	3.2	2.3	++
		0.02 water	2.2	3.6	++++
		0.10 food	2.4	10.8	++++
		0.10 water	2.1	14.0	++++
		1.00 food	1.2	105.0	++++
2-Thiobarbituric acid	$\underbrace{\mathrm{NHCSNHCOCH_2CO}}$	0.002 water	3.9	0.6	+
		0.005 water	3.1	1.3	+
		0.01 water	3.5	3.6	++
		0.10 food	2.9	17.2	++++
Allylisopropyl-*2*-thiobarbituric acid	$\underbrace{\mathrm{NHCSNHCOC(C_3H_5)(C_3H_7)C}}\mathrm{O}$	0.10 food	2.5	16.4	+
Ethyl (*1*-methyl butyl)-*2*-thiobarbituric acid	$\underbrace{\mathrm{NHCSNHCOC(C_2H_5)(C_5H_{11})C}}\mathrm{O}$	0.10 food	2.4	17.1	–
Phenylethyl-*2*-thiobarbituric acid	$\underbrace{\mathrm{NHCSNHCOC(C_2H_5)(C_6H_5)C}}\mathrm{O}$	0.10 food	1.6	18.5	++
Ethyl-*sec*-butyl-*2*-thiobarbituric acid	$\underbrace{\mathrm{NHCSNHCOC(C_2H_5)(C_4H_9)C}}\mathrm{O}$	*0.10* water	2.3	*14.0*	+
		0.10 food	2.8	17.1	++
		0.40 food	0.6	34.0	–
Benzylthiobarbituric acid	$\underbrace{\mathrm{NHCSNHCOCH(CH_2C_6H_5)C}}\mathrm{O}$	0.10 food	3.4	16.3	–
		1.00 food	2.7	92.0	+
n-Butylallyl-*2*-imino-*2*-thiobarbituric acid	$\underbrace{\mathrm{NHCS(NH)NCOC(C_4H_9)(C_3H_5)C}}\mathrm{O}$	0.10 food	3.0	15.7	+

were considerably more toxic. Phenylthiohydantoic acid was only slightly effective when given at the relatively high dose of 67 mgm. per 100 grams per day. It is also of interest to note that methylisothiourea sulfate was much less active than thiourea. This may indicate that the diamide structure contributes to the activity of thiourea. However, it is somewhat surprising to note that 2-aminothiazole is quite active even though the sulfur atom is a part of the cyclic structure.

Of all the compounds shown, 2-thiouracil and 2-thiobarbituric acid were the most active; 3 to 4 mgm. daily induced maximal responses. The toxicity of these two compounds was low; a daily intake for 10 days of 100 mgm. was well tolerated.

The derivatives of 2-thiobarbituric acid such as those used for their hypnotic or anaesthetic properties were much less active on the thyroid than 2-thiobarbituric acid itself.

2-Thiouracil has been more extensively investigated than any other compound in this series. It is slightly more effective than 2-thiobarbituric acid, and toxic manifestations do not occur until a daily dose of 100 mgm. per 100 grams of body weight is exceeded When this compound was fed at a level of 1% in the food, concretions in the urinary tract were noted in about one third of the animals. Animals surviving this complication have been maintained on this dosage for five months, and during this time showed no symptoms or signs other than those attributable to a state of hypothyroidism. This dosage amounts to more than 100 times the minimal effective dose for thyroid hyperplasia and about 20 times the amount required for a full thyroid effect.

Derivatives of Aniline. In table 3 are shown 26 compounds that are chemically unrelated to those shown in table 2 Of these, 13 were found to be effective. The only feature that is common to each of this second series of active compounds is the aniline group. Para aminobenzoic acid is the simplest substance in this series. A daily dose of about 200 mgm per 100 grams is needed for a full effect, and a similar order of activity is possessed by *m*-aminobenzoic acid, *p*-aminoacetanilide, *p*-aminophenylacetic acid and sulfanilamide. The derivatives of sulfanilamide are likewise active Sulfapyridine induced a maximal response with a daily dose of 64 mgm. per 100 grams body weight and sulfadiazine, the most active substance in this group, with a dose of 23 mgm.

Aniline itself was highly toxic and gave no thyroid response with the very small dose tolerated. Several relatively non-toxic aniline derivatives were not active, e g. *p*-aminophenol, *p*-phenylenediamine, *p*-acetamidobenzoic acid, ethyl *p*-aminobenzoate, acetophenitidin and procaine, indicating that some further character is essential to the thyroid effect besides the aniline group. Activity is greatly decreased or abolished altogether when the amino group is substituted as in *p*-acetamidobenzoic acid and *p*-dimethylaminobenzaldehyde.

Cyanides and Thiocyanates. In addition to these two series of compounds, tests were performed on thiocyanates and organic cyanides, as members of this class of compounds have been reported to cause goiter in experimental animals. Methyl cyanide, cyanacetamide, and cyanamide gave negative results at all

TABLE 3

Aniline derivatives[1]

COMPOUND	FORMULA	METHOD OF ADMINISTRATION	DAILY BODY WEIGHT GAIN	AVERAGE DOSE	THYROID RESPONSE
		%	grams	mg./100 gm./day	
p-Aminobenzoic acid	$NH_2C_6H_4COOH$	0.50 water	3.5	180.0	++
		1.00 water	2.9	266.0	++++
		2.00 water	2.3	410.0	++++
m-Aminobenzoic acid	$NH_2C_6H_4COOH$	1.00 water	3.0	228.0	++
		2.00 water	2.6	574.0	+++
o-Aminobenzoic acid	$NH_2C_6H_4COOH$	1.00 water	3.0	269.0	+
		2.00 water	1.8	396.0	++
p-Aminophenylglycine	$NH_2C_6H_4NH(CH_2)COOH$	0.25 water	0.9	82.0	–
p-Aminophenol	$NH_2C_6H_4OH$	1.00 food	1.0	144.0	–
Aniline	$NH_2C_6H_5$	1.00 water	–3.0	11.0	–
p-Phenylenediamine	$NH_2C_6H_4NH_2$	1.00 food	–0.3	137.0	–
Benzidine	$NH_2C_6H_4C_6H_4NH_2$	1.00 food	died		–
		0.50 food	–2.3	22.0	–
p-Aminoacetanilide	$NH_2C_6H_4NHCOCH_3$	2.00 food	0.2	199.0	+++
p-Acetamidobenzoic acid	$CH_3CONHC_6H_4COOH$	1.00 water	2.1	304.0	–
		2.00 water	0.1	457.0	–
Ethyl *p*-aminobenzoate	$NH_2C_6H_4COOC_2H_5$	1.00 food	2.3	120.0	–
		2.00 food	0.1	251.0	–
p-Dimethylaminobenzaldehyde	$(CH_3)_2NC_6H_4CHO$	1.00 food	1.0	90.0	+
p-Aminophenylacetic acid	$NH_2C_6H_4CH_2COOH$	0.10 water	3.7	30.0	+
		1.00 water	2.7	236.0	+++
Sulfanilic acid	$NH_2C_6H_4SO_3H$	2.00 water	2.8	584.0	–
2-Aminotoluene-*4*-sulfonic acid	$NH_2C_6H_3(CH_3)SO_3H$	1.00 water	3.3	175.0	–
		2.00 food	2.7	250.0	–
Procaine	$NH_2C_6H_4COOCH_2CH_2N(C_2H_5)_2$	1.00 food	1.5	119.0	–
		2.00 food	died		
Acetophenetidin	$CH_3CONHC_6H_4COC_2H_5$	2.00 food	–0.7	151.0	–
Guanylsulfanilic acid	$NH_2C(NH)NHC_6H_4SO_3H$	2.00 food	3.0	218.0	–
p-Toluenesulfonanilide	$CH_3C_6H_4SO_2NHC_6H_5$	1.00 food	2.4	118.0	–
Sulfanilamide	$NH_2C_6H_4SO_2NH_2$	0.10 water	2.6	29.4	–
		0.50 water	2.0	70 4	+
		2.00 food	0.9	206.0	+++

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[1] Some of the data on the sulfonamides were taken from a previous publication (5).

TABLE 3—*Concluded*

COMPOUND	FORMULA	METHOD OF ADMINISTRATION	DAILY BODY WEIGHT GAIN	AVERAGE DOSE	THYROID RESPONSE
		%	grams	mg /100 gm /day	
Sulfanilylurea	$NH_2C_6H_4SO_2NHCONH_2$	0 20 food	3 4	22 5	+
Sulfaguanidine	$NH_2C_6H_4SO_2NHC(NH)NH_2$	0 10 water	2 6	22 3	+
		0 50 food	2 6	51 0	++
		2 00 food	2 9	268 0	+++
		5 00 food	2 3	735 0	++++
Sulfathiazole	$NH_2C_6H_4SO_2NHC_3H_2NS$	0 10 water	3 0	21 7	+
		0 50 water	2 8	69 0	++
		1 00 food	2 4	106 0	+++
Sulfapyridine	$NH_2C_6H_4SO_2NHC_5H_4N$	0 10 water	2 9	18 9	+
		0 50 water	2 2	64 3	++++
		1 00 food	1 9	98 0	++++
Sulfadiazine	$NH_2C_6H_4SO_2NHC_4H_3N_2$	0 01 water	3 7	2 4	+
		0 02 water	2 6	4 2	+
		0 10 water	3 4	22 8	++++
		1 00 food	1 5	119 0	++++
Sulfasuxidine	$HOOC(CH_2)_2CONHC_6H_4SO_2NHC_3H_2NS$	5 00 food	3 8	760 0	++

TABLE 4

Cyanides and thiocyanates

COMPOUND	FORMULA	METHOD OF ADMINISTRATION	DAILY BODY WEIGHT GAIN	AVERAGE DOSE	THYROID RESPONSE
		%	grams	mg /100 gm /day	
Methyl cyanide	CH_3CN	1 00 water	−2 9	39 0	−
Cyanacetamide	$CH_2(CN)CONH_2$	0 10 water	2 5	22 9	−
Cyanamide	NH_2CN	0 10 water	3 8	22 8	−
Sodium thiocyanate	$NaSCN$	0 25 food	1 9	35 1	+†
		0 25 water	2 6	74 5	+
		0 25 water*	2 5	70 5	−
		1 00 water	−1 0	165 0	+++
		1 00 water*	−1 8	146 1	−
Potassium thiocyanate	$KSCN$	1 00 water	−0 3	97 5	+++
Ammonium thiocyanate	NH_4SCN	1 00 water	died	156 1	−
Sodium thiosulfate	$Na_2S_2O_3$	0 50 water	2 7	158 0	−
		2 00 water	0 8	387 0	−
Methyl thiocyanate	CH_3SCN	0 10 water	2 7	13 3	−
Guanidine thiocyanate	$NH_2C(NH)NH_2SCN$	0 10 water	3 1	24 5	−

* Plus 0 1% potassium iodide in the drinking water

† Negative after 7 days, +++ after 28 days

TABLE 5

Inactive compounds

COMPOUND	FORMULA	METHOD OF ADMINISTRATION	DAILY BODY WEIGHT GAIN	AVERAGE DOSE
		%	*grams*	*mg./100 gm./day*
Methylurea	$NH_2CONHCH_3$	2.00 food	1.5	249.0
p-Tolylurea	$NH_2CONHC_6H_4CH_3$	1.00 food	1.4	71.5
Guanidine hydrochloride	$NH_2C(NH)NH_2HCl$	0.50 food	1.3	64.0
Nitroguanidine	$NH_2C(NH)NHNO_2$	2.00 food	1.4	223.0
Guanylurea *n*-sulfonic acid	$NH_2C(NH)NHCONHSO_3H$	2.00 food	2.4	117.0
Uracil	$\underline{NHCONHCOCHCH}$	0.10 water	3.5	24.2
2-Aminopyrimidine	$\underline{NC(NH_2)NCHCHCH}$	0.50 water	−0.8	50.9
Isocytosine	$\underline{NHCONCHCHC}NH_2$	0.10 water	3.5	18.7
Acetoguanamine	$\underline{C(NH_2)NC(NH_2)NC(CH_3)N}$	0.10 water	2.3	19.0
Benzoguanamine	$\underline{C(NH_2)NC(NH_2)NC(C_6H_5)N}$	0.10 water	1.6	13.6
2-Pyridylthioacetic acid	$\underline{NC(CH_2CSOH)CHCHCHCH}$	0.10 water	1.4	14.6
2-Aminobenzothiazole	$\underline{SC(NH_2)NC}\overline{HCHCHCHCHC}H$	0.10 water	0.9	11.7
Trithiomethylene	$\underline{CH_2SCH_2SCH_2S}$	0.20 food	1.6	7.8
		1.00 food	1.2	90.5
Sulfonmethane	$(CH_3)_2C(SO_2C_2H_5)_2$	0.30 water	2.2	62.1
Thioacetamide	NH_2CSCH_3	0.10 food	−1.8	0.5
Thiobenzamide	$NH_2CSC_6H_5$	0.10 water	−0.2	3.7
Dithiooxamide	$NH_2CSCSNH_2$	1.00 food	died	23.5
		0.20 food	−0.7	12.8
Thiochrome	$C_{12}H_{14}ON_4S$	0.10 water	2.7	16.2
		1.00 food	3.5	106.0
Benzoic acid	C_6H_5COOH	2.00 water	−0.2	426.0
Sulfosalicylic acid	$HOOCC_6H_3(OH)SO_3H$	1.00 food	3.5	155.0
Saccharin	$\underline{C_6H_4SO_2NHCO}$	1.00 food	3.4	157.0
Benzenesulfonamide	$C_6H_5SO_2NH_2$	0.10 water	0.8	12.2
p-Toluenesulfonamide	$CH_3C_6H_4SO_2NH_2$	1.00 food	1.1	104.0
Phenolsulfonic acid	$HOC_6H_4SO_3H$	0.10 water	3.4	24.7
Dinitrophenol	$C_6H_3(NO_2)_2OH$	0.10 water	1.5	9.9
n-Butyl sulfone	$SO_2(CH_2CH_2CH_2CH_3)_2$	0.10 water	1.8	12.0
Diphenyldisulfide	$C_6H_5S—SC_6H_5$	0.20 food	−0.3	15.1
Sulfur	S	5.00 food	1.9	604.0
Sodium sulfide	Na_2S	0.50 water	3.7	179.0
Ammonium sulfamate	$NH_2SO_3NH_4$	1.00 water	2.4	195.0
Hydroquinone	OHC_6H_4OH	2.00 food	3.1	290.0
Methylene blue	$C_{16}H_{18}N_3SCl$	0.50 water	−2.4	56.0
8-Hydroxyquinoline	$\underline{C_6H_3(OH)NCHCHC}H$	2.00 food	2.0	245.0
Sodium sec. butyl xanthate	$CH_3CH_2CH(CH_3)OCSSNa$	0.10 water	1.7	16.9
Sodium diethyl-dithiocarbamate	$(C_2H_5)_2NCSSNa$	1.00 food	1.3	111.0
Ascorbic acid	$C_6H_8O_6$	1.00 water	0.2	238.0
Tyrosine	$HOC_6H_4CH_2CH(NH_2)COOH$	0.50 water	2.1	179.5
3,5-Diiodo-*4*-hydroxybenzoic acid	$HOC_6H_2I_2COOH$	1.00 water	−1.6	46.6
p-Nitrobenzoic acid	$NO_2C_6H_4COOH$	1.00 water	−0.6	111.0

dosage levels (table 4). The earlier tests on the thiocyanates gave inconstant and poorly reproducible results but later it became clear that at least two factors modified the thyroid response to these agents. On the usual diet of Purina fox chow marked thyroid hyperplasia resulted when either sodium or potassium thiocyanate was administered over periods of several weeks. When given for a shorter period the result was less constant and little or no hyperplasia was to be seen in animals killed one week after the beginning of treatment. Thus a thyroid effect was much slower to develop with the thiocyanates than with the thioureas and aniline derivatives. The administration of iodide together with sodium thiocyanate completely abolished the thyroid hyperplasia and no thyroid change was noted even when treatment was continued for 3 weeks. As iodide in large doses does not modify the thyroid response to compounds of the thiourea and aniline series, this peculiarity of the thiocyanates indicates that a different type of action is involved.

Miscellaneous Inactive Substance. A variety of compounds that have yielded negative results are shown in table 5. This list includes derivatives of urea and guanidine, compounds containing sulfur and substances related in one way or another to those shown in tables 2 and 3.

Discussion. The method used to determine the antithyroid activity of the above compounds at best provides an estimate of the relative activities of the several substances tested, and the figures obtained cannot be considered as quantitatively accurate. A number of factors enter which make for error. Measurements of the daily food and water intake are subject to small errors, and losses by the scattering of food and the evaporation of the drinking water result in an overestimate of the quantity of drug actually consumed. Probably the greatest error occurs in the tests on toxic substances and on compounds that are disagreeable to the taste. It is clear that the thyroid response is distinctly less in animals which are ill and in those which fail to gain weight because of decreased food or water intake. This is exemplified in the experiments on ethyl-*sec*-butyl-thiobarbituric acid and phenylthiohydantoic acid in which an increase in the dose to toxic levels resulted in a lessening of the thyroid response. It is quite probable that the antithyroid activity of some compounds is entirely eclipsed by their toxic effects; guanylthiourea, *o*-tolylthiourea, dithiooxamide and dithiobiuret may be examples of this. However this method may be considered adequate to the purpose for which it was intended. It is sufficiently accurate for a first approximation of the activity of widely different chemical compounds of unknown potency and has the distinct advantage of being simple and reproducible. Administration of drugs by their incorporation in the diet provides for a relatively constant rate of absorption, a factor of importance when chronic effects are being observed. Furthermore it is of special practical interest and clinical importance that substances be found which are active when administered by mouth.

The rate of body growth in young animals is one of the most sensitive tests of toxicity that can be used. However it is only reliable in a negative sense; substances which are disagreeable to the taste will likewise decrease the rate of growth because of lowered food consumption. Therefore the figures given above for the rate of growth are of importance chiefly in indicating which compounds are non-toxic.

The structures of the compounds that proved to be active in this test indicate that two general classes of substances possess this type of physiological action. All of the compounds having within their structure the grouping —NH·CS·NH— were active with the exception of those of very low solubility in water or of high toxicity. The activity of thiourea itself was not perceptibly altered by the presence of a methyl, acetyl or allyl group; the symmetrically substituted thioureas were all less active except the diethyl derivative which was about twice as active as thiourea. Most of these compounds were more toxic than thiourea however. The inclusion of thiourea in a five or six membered heterocyclic structure resulted in a pronounced enhancement of activity as in the thiohydantoins, thiobarbituric acid and thiouracil. However the six membered heterocycles containing 3 nitrogen atoms such as thioammeline, dithioammelide and monothiocyanuric acid, although non-toxic in large doses, were of very low potency. Most of the heterocycles were of low toxicity and thiouracil and thiobarbituric acid in addition to exhibiting an unusually high activity were particularly non-toxic and therefore of greatest interest clinically.

From a structural point of view it is apparent that the entire grouping —NH·CS·NH— is essential to the thyroid effect. Activity is lost if the S is replaced by another element or group as in urea, guanidine, and their derivatives, uracil, *2*-aminopyrimidine, isocytosine and the guanamine derivatives. Apparently both amino nitrogen atoms are essential, for activity is lost when one of these groups is absent as in thioacetamide, thiobenzamide, sodium *sec.*-butylxanthate and sodium diethyldithiocarbamate. The sulfur itself is obviously not the active agent as a number of compounds containing sulfur in different forms were inactive. Therefore, as far as this series of experiments go, it appears that the entire thiourea grouping is essential to the type of biological response under investigation.

The biologically active grouping in the second series of compounds is not entirely clear, and the only structure common to all is the aniline group $NH_2 \cdot C_6H_4$—. Substances closely related to certain of the active aniline derivatives were inert, e.g. benzoic and sulfanilic acids, benzene- and toluenesulfonamides, phenylsulfonic acid and saccharin. It is apparently necessary that the amino group be free, for activity was lost when this group was substituted as in *p*-acetamidobenzoic acid, and *p*-dimethylaminobenzaldehyde. Aminobenzoic acid was most active when the carboxyl group was in the para position to the amino group. Compounds possessing the same order of activity as *p*-aminobenzoic acid were: *p*-aminoacetanilide, *p*-aminophenylacetic acid, and sulfanilamide. Potency was increased when one of the hydrogen atoms of the sulfamido N of sulfanilamide was replaced by a guanyl-, thiazole-, pyridine- or pyrimidine grouping, and in the case of sulfadiazine the activity approached that of the active members of the thiourea series of compounds. It is possible that these aniline derivatives owe their activity to their structural similarity to tyrosine It will be of interest to determine the activity of compounds related to tyrosine or diiodotyrosine in which the *p* hydroxyl group is replaced by an amino group as in *p* aminophenylalanine

As a working hypothesis it is suggested that the aniline derivatives act through a competitive mechanism in the enzyme system responsible for the conversion of diiodotyrosine to thyroxin The thioureas are possibly specific inhibitors of this same system

SUMMARY

The relative effectiveness of 106 chemical compounds in inhibiting the function of the thyroid gland was tested in young rats When these substances were administered in the food or drinking water for a period of ten days, the degree of thyroid hyperplasia, as estimated by gross and microscopic examination, provided an estimate of their activity

Two classes of compounds were found to be active thiourea derivatives and certain derivatives of aniline The compounds more effective than thiourea in decreasing order of activity were *2* thiouracil, *2* thiobarbituric acid, *sym* diethylthiourea and *5* benzal *2* thiohydantoin The effective aniline derivatives comprised the sulfonamides, *p* , *m* , *o* aminobenzoic acids *p* aminophenyl acetic acid and *p* aminoacetanilide Thiocyanates were found to cause thyroid enlargement only in the absence of added iodide, while organic cyanides failed to influence the thyroid gland

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THE EFFECT OF HIGH FAT DIET ON CHRONIC TOXICITY OF DERRIS AND ROTENONE[1]

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It has been reported by Lightbody and Mathews (1) that when rotenone is dissolved in olive oil and administered gastrically to rats and guinea pigs its toxicity is greater than when administered in water suspensions. Ambrose and Haag (2) reported the same observations, and also demonstrated in rats and rabbits that gastric administration of a water suspension of derris followed immediately by olive oil resulted in augmentation of the toxicity.

These interesting observations on the role of fat in the toxic action of rotenone and derris are of practical significance chiefly because they reveal a source of error in the determination of the acute toxicity of these substances. Interest in the acute toxicity of rotenone and derris in warm blooded animals is concerned with the translation of the data in terms of possible toxic dosage in humans. Since there is only a remote possibility of acute poisoning being associated with high fat intake in humans, the acute toxicity data involving use of olive oil gives an exaggerated idea of toxicity.

However, a different situation presents itself when public health hazards traceable to derris spray residues are considered. In the absence of experimental evidence arguments could be advanced to show that individuals using a high fat diet might be either more or less susceptible to effects of chronically ingested derris. Lightbody and Mathews (1) have reported experiments on chronic ingestion of rotenone extending over a period of 37 days, at the end of which time the last experimental animal died. Their data are unquestionably correct under the experimental conditions employed. They dissolved all doses of rotenone in olive oil in such concentrations that all rats received daily 2.5 cc. of olive oil per kilogram of body weight, administered by stomach tube. Dissolved in olive oil the rotenone is in a high degree of dispersion, a condition which, in conjunction with the mode of administration employed, does not simulate the circumstances under which humans on a high fat diet would be subjected to a spray residue hazard involving rotenone or derris.

The present report gives data on the continued feeding of derris and rotenone to albino rats maintained on diets in which the fat content had been raised by incorporating Crisco. The derris (sample No. I. D. 3006, containing 3.6% rotenone and 15.6% carbon tetrachloride extractives) and rotenone employed were supplied by the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture. Both the derris and rotenone were fine enough to pass through a 100 mesh sieve. Male albino rats weighing approximately 45 grams each were used in these feeding experiments. The rats were divided into 10 groups of 5 rats each and placed on the following diets:

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[1] Agricultural Chemical Research Division Contribution No. 93.

Group 1—Control diet
Group 2—Control diet plus .03% derris
Group 3—Control diet plus 20.0 % Crisco
Group 4—Control diet plus .03% derris and 15% Crisco
Group 5—Control diet plus .03% derris and 10% Crisco
Group 6—Control diet plus .03% derris and 5% Crisco
Group 7—Control diet plus .03% derris and 2.5% Crisco
Group 8—Control diet plus .06% derris and 15% Crisco
Group 9—Control diet plus .008% rotenone
Group 10—Control diet plus .008% rotenone and 15% Crisco

In all cases the concentration of derris or rotenone employed refers to the final concentration in the completed diet.

These diets were placed in food cups designed to reduce food losses to a minimum. The cages were provided with water bottles, and the rats had free access to food and water at all times. The rats and food cups were weighed once a week, at which time careful observations were made as to the general condition of the animals. All rats were kept on their respective diets for at least 140 days. Upon termination of the feeding experiments all rats were autopsied and observations were made for evidence of gross changes The liver, spleen, kidney, adrenal, heart, bladder, intestine, lung, testis, and stomach were examined for evidence of histological changes.

The various diets were prepared in the following manner. Diets 2 and 9 were prepared by adding the weighed amount of derris or rotenone and thoroughly mixing in a mixing machine. Diet 3 was prepared by adding the weighed amount of Crisco to the diet, mixing roughly by rubbing in the hands, and finally securing even distribution of the fat in the mixing machine Diets 4 to 8, inclusive, were made by incorporating the derris as in diet 2 and then adding the desired amount of Crisco as in diet 3 Diet 10 was prepared by adding the rotenone to the melted fat and incorporating the mixture in the basic diet in the mixing machine.

Comparison of the growth rates of the three control groups receiving the basic diet, basic diet plus .03 per cent derris, and basic diet plus Crisco, with those of the animals receiving derris together with Crisco, showed that only in the animals of group 8, which received .06 % derris and 15% Crisco was there a significant decrease in growth rate. However, the retardation of growth was no greater than that previously reported (3) for a diet containing .06% derris, and the effect was attributed solely to the derris. The slight variations in growth rates of groups receiving .03% derris in combination with various concentrations of fat in the basic diet showed no consistent relationship to the percentage of Crisco employed. So far as the criterion of growth is concerned, fat in the form of Crisco in concentrations ranging from 2.5 to 15% did not potentiate the chronic toxicity of derris

Rats receiving a diet containing .008% rotenone and 15% Crisco showed a moderate retardation of growth for the first 35 days of the experiment as compared with the growth of rats eating a diet containing .008% rotenone. Subsequently, the growth rates of the two groups were almost identical. The level of rotenone intake corresponded to approximately 5 milligrams per kilogram of body weight per day, the equivalent of 139 milligrams of derris per kilogram of body weight per day for a sample of derris containing 3 6% rotenone. As in the case of the experiments involving derris it is evident that a high fat diet did not produce any pronounced potentiation of the toxicity of rotenone.

Despite the variations in the caloric value of the control diet and the diets containing Crisco the records of food intake do not show that a high fat content was associated with a decreased food consumption. For example, rats eating the basic diet containing .03% derris consumed an average of 59.6 grams of food per kilogram of body weight per day, corresponding to .018 grams of derris, and rats eating the diet containing .03% derris and 15% Crisco consumed 57.4 grams of food per kilogram of body weight per day, coresponding to .017 grams of derris. This observation agrees with the fact that the slight variation in growth rates of groups receiving .03% derris in combination with various concentrations of Crisco in the basic diet showed no consistent relationship to the amount of Crisco used.

We have previously reported (3) that even when growth curves revealed no evidence of a chronic toxic action of derris the histological examination of stained tissue sections showed definite evidence of liver injury. It was therefore necessary to apply the same criterion to the present studies. The tissues were imbedded in paraffin, sectioned and stained with hemotoxylin and eosin. Practically no changes were noted in the liver, spleen, kidney, adrenal, heart, bladder, intestine, lung, testis, or stomach of any of the animals receiving derris and Crisco. Several showed a mild gastritis unrelated to the size of the dose of derris. In the convoluted tubular epithelium of the kidneys from the rats receiving all of the above combinations of derris and Crisco there were a few scattered small deposits of brown granular pigment. The livers showed no changes which can be considered due to the toxic action of derris.

As previously reported (3), the livers of rats receiving a diet containing .03% derris without added fat showed histological evidence of liver damage. The absence of liver damage in rats receiving derris in a high fat diet suggests that fat in the form of Crisco has reduced toxicity, possibly by interference with absorption. These results are in striking contrast with those obtained in acute experiments where fat in the form of olive oil augmented the toxicity. It is true that the concentrations of derris and rotenone employed were low as compared with the amounts of rotenone employed by Lightbody and Mathews, but they were well within the range of derris concentrations which we have shown will produce liver damage. The results emphasize the fact that one cannot predict chronic toxicity on the basis of acute toxicity data.

SUMMARY

Albino rats have been maintained on high fat diets containing derris and rotenone for a minimum of 140 days. Growth curves failed to show any appreciable retardation of growth of the experimental rats as compared with control rats. Histological examination of the tissues failed to show any evidence of tissue damage.

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A METHOD FOR STUDYING THE ANALGETIC EFFECT OF DRUGS IN ANIMALS

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Quantitative pharmacological studies of analgetic drugs have been handicapped by the lack of accurate measurement of the pain stimulus used. Many methods have been proposed, using pressure, electrical or heat devices to evoke pain responses. Perhaps the most satisfactory method at the present time is that of Hardy, Wolff and Goodell (1). This procedure adapts itself very readily to man, but is less applicable for animal studies. While it has been used on animals (2) with a muscle reflex as the index of pain sensation, it requires that the animal be trained to lie in a fixed position, otherwise the focal range becomes disturbed.

Several forms of electrical stimuli have been employed. Martin (3) employed a liquid electrode applied to the finger, but this method again limits the studies to man. Macht (4) also used the principle of this method in his studies of the opiates by applying small platinum electrodes to various areas of the body. The measurements were recorded with a calibrated induction coil. Recently Lanier (5) has published a technique similar to that reported in this paper. While the measurement of pain in animals presents greater difficulties than encountered in man, the desirability of such measurements is obvious.

In a search for an electrical method to produce cutaneous pain in animals, three problems presented themselves. First, the proper type of stimulus; second, the application of the stimulus; and thirdly, what sign or signs are to be used as an indication of recognition of pain by the animal.

Method. The stimulus used was repetitive condenser discharge. The intensity, frequency, pulse duration and duration of application could be varied independently. The stimulus as used in these experiments was a pulse duration of 0.4 millisecond at a frequency of 200 per second applied for 0.5 second. The stimulus strength in milliamperes was calculated from the peak voltage measured across a calibrated resistance, placed in series with the stimulating electrodes. A cathode ray oscillograph was used as a peak voltmeter. For the sake of accurate measurement, the standing wave produced by having the sweep circuit of the oscillograph synchronized with the stimulus rate was photographed and measurements made from the photograph. An interval of thirty seconds was allowed between applications of the stimuli, to prevent the fatigue of the pain receptors.

The electrodes used were one large indifferent electrode of about two square inches, smeared with electrode jelly and fixed in position on a shaved area of the dog's leg. The other electrode consisted of thirty pin heads soldered into a metal plate of 1¼ inch square. Von Frey (6) has shown the presence of definite pain points scattered more or less abundantly over different parts of the body. The use of the multiple pin head electrode insures contact with a reasonable number of pain points. The use of a solid electrode over 0.5 sq. cm. was found to be unsatisfactory because it produced powerful muscle contractions. The multiple point electrode was fixed in position over a shaved area of the back of the animal so that firm contact was made without exerting undue pressure. The tension of this electrode was controlled by having it attached to an adjustable steel spring. If excessive pressure was employed the pain points fatigued rapidly and no consistent measurements could be obtained. To insure that the same area was stimulated in each individual experiment, the skin area was outlined with a skin pencil.

Dogs require very little training to be sufficiently quiet on a table for the experimental procedure and it does not require the exactness of position of the Hardy-Wolff method. However, the animal should be placed in a separate room so that its attention is not distracted by the apparatus. This can be easily accomplished by wire leads from the apparatus to the separate room.

The index for recognition of the stimulus finally adopted, was an opening or widening of the eyelids. This is fairly reliable and an early sign of recognition. If the intensity of the stimuli is increased slightly over that required to cause a widening of the eyelids, the dog raises his head and a still greater intensity may cause vocalization. While the only sure sign of pain in an animal is a typical cry or howl, the sequence of events was so uniform that we believe the eyelid response to be a true pain threshold recognition sign. This sign could be elicited over several hours with very little variation in the intensity of stimuli, providing the same skin area was stimulated.

It was found that the muscle reflex or skin twitch as used in the Hardy-Wolff method (2) could not be used as an index of threshold recognition. While this muscle twitch appears to be a reliable index with the thermal stimulus, it is not altered by the analgetic drugs when the electrical stimulus method is used.

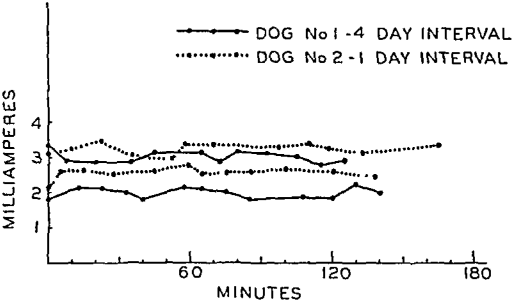
While the method as described has only been used on dogs, it could probably be adapted to other animals.

EXPERIMENTAL. Since we were primarily interested in testing the validity of the proposed method for determining quantitatively the analgesic action of drugs, we employed for the most part only substances with proven potency.

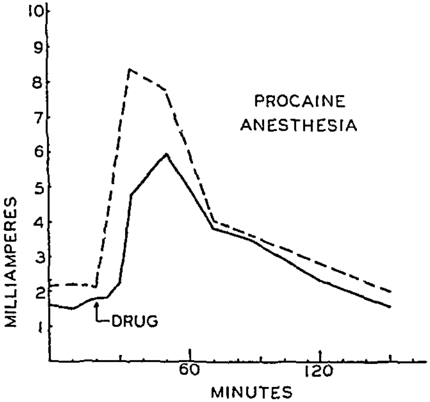
Control determinations were made on two dogs, but only one dog was used for the administration of analgetic drugs. This dog was an animal with a stomach fistula and had been previously trained to lie quietly on the table. All the oral administrations were made directly into the stomach through the fistula by means of a catheter. With the exception of one experiment with alcohol, all the drugs were given on an empty stomach. We made no attempt to place the multiple point electrode in exactly the same spot in different experiments. Therefore after the electrodes were fixed in position, control evaluations of recognition were made at the start of each experiment (see graphs). It will be noted that the initial thresholds vary considerably, probably due to the fact that we were stimulating somewhat different skin areas in each of the experiments. Several control experiments were made on the two dogs and it was found that when the same skin area was stimulated, fairly constant readings over several hour periods could be obtained with maximum variations of about 0.5 milliamperes (graph 1).

Local Anesthesia. In order to determine whether the skin twitch was a nerve or direct muscle stimulation, the skin area under the multiple electrode was infiltrated with 5 cc. of 2% procaine. If the skin twitch was due to direct muscle action, it should not be affected by the local anaesthetic, as there is no proven evidence that this anasethetic directly alters muscle irritability. (Graph 2).

It was found after local anaesthetization that the stimuli required to elicit the recognition sign and the skin twitch were markedly elevated, although there



GRAPH 1 CONTROL CURVES OF TWO DOGS ARE PRESENTED TO SHOW THE NORMAL VARIATIONS IN RECOGNITION THRESHOLD



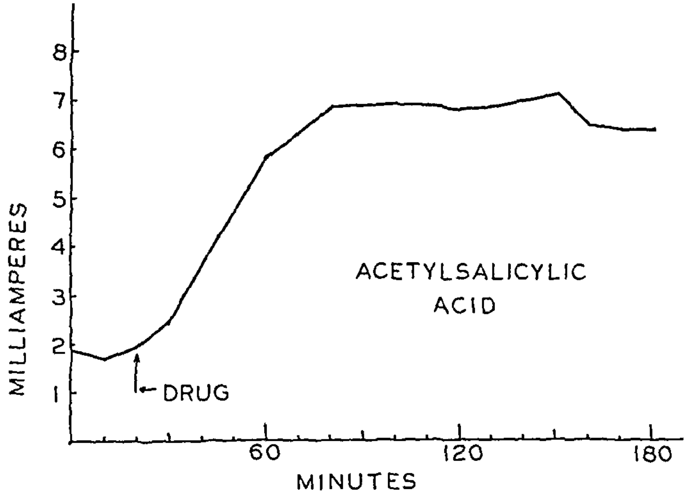
GRAPH 2 EFFECT OF PROCAINE ANESTHESIA ON THE RESPONSE TO ELECTRICAL STIMULI
Solid line = skin twitch Broken line = recognition threshold

was a longer latent period for the skin twitch. Similar infiltration with 5 cc. of distilled water had no effect in altering either the recognition sign or skin twitch after the first five minutes.

Acetyl Salicylic Acid. Two experiments were performed on the effect of acetyl salicylic acid in increasing pain threshold. Two five grain tablets of aspirin were powdered and washed into the stomach with a small amount of water (graph 3).

As the results of these two experiments were very similar only one curve is presented. It will be noted that there is a fairly sharp rise in the recognition threshold, which reaches a peak in about 80 minutes. The intensity of the stimulus to produce recognition was noted at intervals for 190 minutes. One reading made at the end of 480 minutes showed that the threshold had returned to approximately normal level.

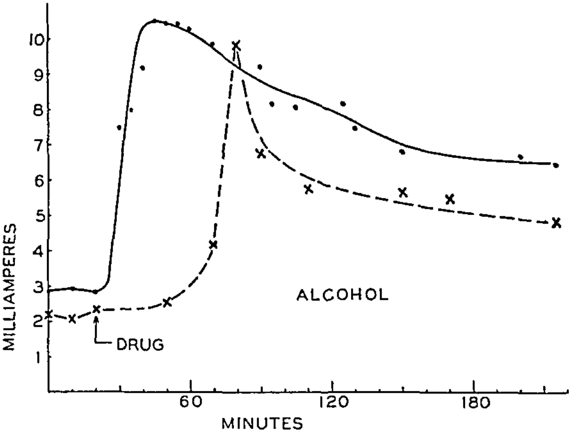
Alcohol. Two experiments with alcohol were performed, one with 30 cc. of alcohol diluted to 120 cc. given on an empty stomach, the other with 20 cc. of alcohol diluted to 100 cc. given one hour after feeding. In the first experiment it will be noted that there is a very sharp rise in the recognition threshold reaching a peak in 26 minutes and then gradually declining (graph 4).



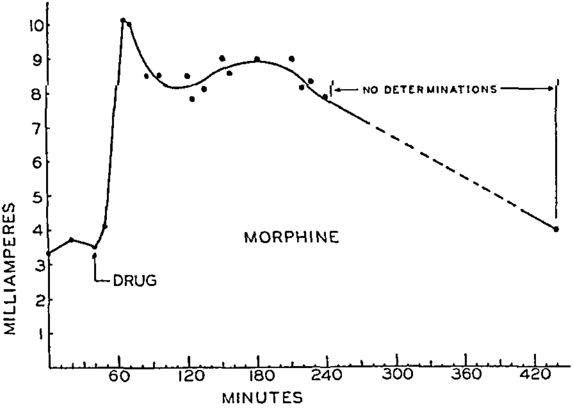
Graph 3. Effect of Acetyl Salicylic Acid
(About 600 mgm.—10 grains.) Approximately 40 mgm. per kgm.

In the second experiment, influence of food in delaying absorption is demonstrated by the slow increase in threshold for the first 50 minutes, followed by a sharp rise. We have no explanation for the sudden fall in the threshold of this animal after the initial peak. No observations were made beyond 190 minutes. The animal was still definitely under the influence of alcohol at the end of this period, as it had poor coordination in walking when removed from the table.

Morphine Sulfate. Two experiments with morphine as the analgetic agent were performed. In the first experiment we used 2.5 mgm. of morphine sulfate per kgm. subcutaneously. This dosage produced a peak threshold beyond the range of our stimulator and was discontinued (graph 5).



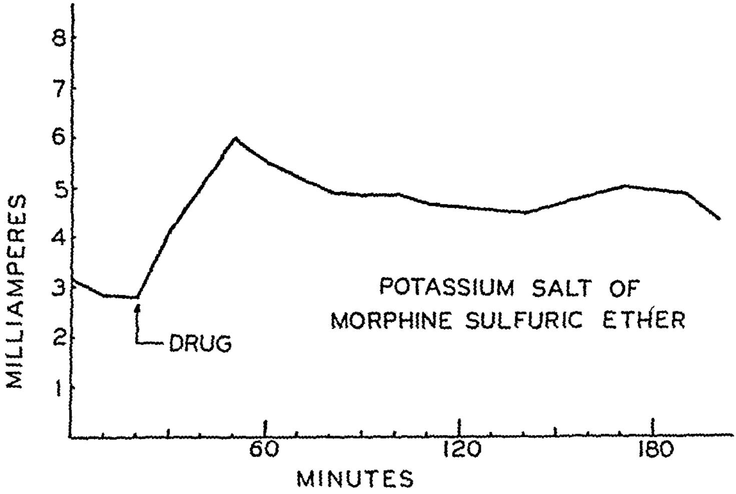
Graph 4 Effect of Alcohol
Solid line = 30 cc of alcohol (2 cc per kgm) diluted to 120 cc given after fasting Broken line = 20 cc of alcohol (1 33 cc per kgm) diluted to 100 cc given one hour after food



Graph 5 Effect of Morphine Sulfate
Total dosage 7 5 mgm (0 5 mgm per kgm, subcutaneously)

In the second experiment we administered 0.5 mgm. per kgm. subcutaneously. The peak threshold was reached in 27 minutes, then declined rather sharply. There was a second rise after 90 minutes which lasted about one hour and then started to fall rather sharply. Periodic observations were made for 200 minutes when the dog was returned to the cage. The next observation was made at the end of 440 minutes. At this time the reading was essentially normal, so that we do not know how long the analgesia persisted.

Morphine Sulfuric Ether.[1] The only agent of unknown analgetic potency used was morphine sulfuric ether. As this compound is insoluble, the potassium salt was prepared. This salt is freely soluble. Previous studies (7) with this compound showed it has only a slight morphine action. It apparently produces little or no narcosis in dogs, but does show slight analgetic action as evidenced by such crude tests as pinching. However, it does reduce abstinence symptoms in human addicts (8) (graph 6).



GRAPH 6. EFFECT OF THE POTASSIUM SALT OF MORPHINE SULFURIC ETHER
Total dosage 150 mgm. (10 mgm. per kgm., subcutaneously)

The dog was injected subcutaneously with 10 mgms. per kgm. No nausea or narcosis was observed but it did produce a mild degree of analgesia. While the per kgm. dosage of this drug was twenty times that of morphine, the pain threshold increased only about $\frac{1}{3}$ of that observed with the smaller dose of morphine. However, the curve is very similar, there being a steep rise with a rather prompt fall and then a secondary rise.

DISCUSSION. In experiments on dogs using electrical stimuli a large variation in threshold of recognition was found. Day to day observations on the same animal are not constant, although approximately the same skin area was stimulated However, initial threshold was constant over several hours when the same area was stimulated Variations in initial threshold as experienced in the various experiments cited are as follows: 2 10, 1 90, 2 15, 2 85, 3 35 and 3 40 milliamperes Lanier (5) has shown that using electrical stimulation in man there is also a wide variation of threshold from individual to individual

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[1] Morphine sulfuric ether was prepared by Dr. L. A. Small, Head Chemist, National Institute of Health.

In the study of the skin twitch it was found that analgetic drugs failed to alter the threshold for this sign It was definitely proved not to be a direct muscle stimulation as it was altered by local anaesthesia Furthermore it was found that after procaine injection there was a definite latent period in the increase in threshold for the skin twitch in terms of original threshold over that for recognition This suggests that fibers stimulated to give recognition response are of smaller size than those which give the skin twitch response The fibers stimulated to give recognition response must have been afferent Those giving the muscle twitch could have been either afferent or efferent The fact that in the thermal response the muscle reflex and recognition parallel each other, makes it likely that with the electrical stimulation there is activation of motor fibers to skin muscles as well as pain receptors It is a well known fact that sensory fibers are paralyzed by local anaesthetics before motor fibers and this fact would explain the delay in onset of the skin twitch Thus the two phenomena with electrical stimulation do not parallel each other under the influence of analgetic drugs In the thermal method only pain receptors are stimulated (1)

CONCLUSIONS

1 An electrical method suitable for determining ' pain" threshold in animals has been described

2 With the electrical method of stimulation both motor fibers and pain receptors are stimulated, while thermal stimulation affects only pain receptors

3 A test of the method using drugs of proven analgetic potency shows that this method is satisfactory to test quantitatively the pain threshold in animals

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ANTIPRESSOR EFFECTS OF ORTHOQUINOID EPINEPHRINE DERIVATIVES IN EXPERIMENTAL HYPERTENSION IN THE RAT[1]

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The experimental induction of hypertension in animals by the production of renal ischemia has been ascribed to reactions similar to the incomplete catabolism of certain aromatic amino acids under anoxic or hypoxic conditions of the kidney, resulting in the accumulation of pressor amines (1, 2). In order to test the validity of this hypothesis, we attempted to counteract these pressor substances by the introduction into the body of chemical compounds designed to inactivate and destroy them. Regardless of the possibility of reconciling the above-mentioned hypothesis with observations on the renin-angiotonin system, the present observations may contribute to the elucidation of the mechanism underlying experimental hypertension in rats.

To clarify the position, substances which reduce the elevated blood pressure to the normal level may suitably be designated as "antipressor" substances, in contrast to "depressor" substances which lower the normal blood pressure. An ideal antipressor substance of possible therapeutic significance should, in the performance of its task, neither possess toxic properties *per se* nor give rise to secondary products with undesirable traits; it should have no depressor effect.

A survey of the experimental facts of pressor amine catabolism (3) shows (*a*) that pressor amines may be attacked in the side chain or in the cyclic portion of their molecule, (*b*) that some intermediate products in either sequence of reactions have antipressor character, thus perhaps adding elasticity to the defense mechanism of the body against flooding with pressor amines, (*c*) that depressor substances may be formed due to secondary reactions, (*d*) that the end products are excretable or inert and, (*e*) that significant differences in pressor amine catabolism may be expected between one animal species and another because of differences in their enzymatic equipment (4).

The oxidation of phenolic pressor amines as well as of other mono- and diphenols by phenolases and catecholases leads to orthoquinoid intermediates, which may either polymerize to melanins or condense with amines, a general reaction of quinones leading to the so-called azophenins. The blood pressure lowering effect of fungus oxidases in experimental and essential hypertension may be attributed both to the simple destruction of pressor amines and to the intermediate production of antipressor orthoquinones (5). The latter mechanism has been brought into play *in vitro* as well as *in vivo*, when mixtures of quinone precursors, aerated in the presence of heavy metal ions, were shown (*a*) to destroy, partly or completely, the effect of pressor amines on the blood pressure of the anesthetized cat and (*b*) to lower the blood pressure of rats, made hypertensive by perinephric scar (6).

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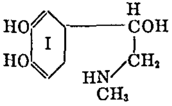
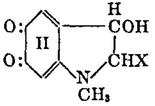
[1] A preliminary report of this work was delivered before the Sections of Medicinal and Biological Chemistry of the American Chemical Society at its Semiannual Meeting in Buffalo, New York, September 8 and 9, 1942.

[2] George Blumenthal, Jr. Fellow.

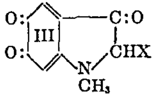
A search for quinones which would answer the requirements of an ideal antipressor substance, enumerated above, led us to the study of orthoquinones of biological origin. The catechol ring, the precursor of the orthoquinone ring, occurs in the animal organism in the epinephrine molecule (I). A very unstable red substance has been obtained by Green and Richter when epinephrine solutions were exposed to the action of mushroom oxidase (7). This product, and similar ones formed by other enzyme systems and believed to be identical, has been designated as adrenochrome and formulated as *N*-methyl-*2*,*3*-dihydro-*3*-hydroxindol-*5*,*6*-quinone (formula II)[3]. More stable compounds may be obtained by the oxidation of epinephrine with iodate (9) or with bromine (7); both are dark crystalline substances with a green lustre, form purplish red solutions, may be reduced to "leuko"-forms, and analyse respectively for *2*-*N*-methyl-iodo- (or bromo-) *2*,*3*-dihydro-*3*-hydroxindol-*5*,*6*-quinone,[4] they have been designated as iodo-adrenochrome and bromo-adrenochrome (II). These products are obtainable from *l*-epinephrine both of natural and of synthetic origin. Drs. J. S. Buck and C. Suter of Winthrop Chemical Company, Rensselaer, New York, have, in addition, prepared for us *d*,*l*-iodo-adrenochrome from racemic epinephrine and the related *N*-methyl-*2*-iodo-*2*,*3*-dihydro-*3*-keto-indole-*5*,*6*-quinone (*1*-methyl-*2*-iodo-*3*,*5*,*6*-triketo-tetrahydroindole) (formula III), a substance of similar appearance as the halogeno-adrenochromes, from adrenalone. The halogeno-adrenochromes, while more stable than their parent substance, may be kept intact



I. Epinephrine



II. X=H: Adrenochrome;
X=I: Iodoadrenochrome;
X=Br: Bromoadrenochrome



III. Triketo-derivatives from adrenalone for a limited time only, and while the methods of preparation described in the quoted references have given to us and others satisfactory yields of material of proper appearance and composition, the keeping qualities of individual batches vary widely in a not yet predictable manner.

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[3] Heirman, (8a), describes the formation of another oxidation product of epinephrine by mushroom oxidase, subsequent to the formation of adrenochrome, and only at pH 7, not at pH 5. This ill-defined product, which he did not isolate and which he terms "adrenoxine," produced bradycardia and displayed depressor properties. Blaschko and Schlossman (8b) were unable to reproduce Heirman's observations on the formation of adrenoxin in the enzymatic preparation of adrenochrome.

[4] The identity of the enzymatic oxidation product with the parent substance of the nonenzymatic halogenated oxidation products is well substantiated. But whereas mushroom, potato and meal worm tyrosinase give rise to "adrenoxine," according to Heirman (8a), "l'oxydation par les oxydants minéraux (permanganate de soude, iodate de potassium) ne la fait jamais apparaître."

The effects of these substances will be described and discussed in the following sections.

Methods. We have used adrenochromes in amounts from 5 to 100 mgm. per rat with 0.5 cc.–1.0 cc. of olive oil or propylene glycol as vehicle. The substances form suspensions in oil, but they dissolve at least partly in propylene glycol. However, according to the degree of decomposition, melanin-like products are present which do not dissolve in propylene glycol.

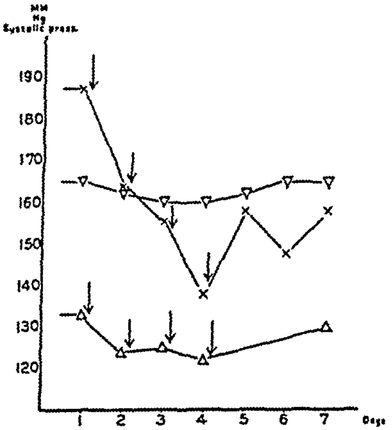

Fig. 1. Effect of Intraperitoneal Injection of 10 Mgm. Iodo-adrenochrome on 4 Successive Days in Hypertensive and Normal Rats

Curve 1. ▽—▽, hypertensive controls without injections (3 animals).
Curve 2. △—△, normal rats (4).
Curve 3. ×—×, hypertensive rats (4).
The arrows indicate days of injection.

The route of administration was by intraperitoneal or intramuscular injection. Neither olive oil nor proplyene glycol, in the same or in larger amounts than those used as vehicles and administered by the same routes, affected the blood pressure of normal or of hypertensive rats.

Hypertension was induced in the animals by wrapping either one or both kidneys with cellophane. Adult male albino rats weighing 250–370 grams were used. Experiments were begun when the blood pressure had reached and maintained for two weeks a plateau at least 40 mm. Hg above the normal blood pressure. The animals were kept on a normal diet *ad lib.*

The blood pressure was estimated by the method of Williams, Harrison, and Grollman (10). The rectal temperature of the rats was recorded and they were placed for at least 10 minutes in a well ventilated heat box kept at 38 ± 1° prior to blood pressure measurement. Three readings were taken once a day and the average recorded. Curve 1 (fig. 1) illustrates the measurements over one week on 3 rats whose combined average blood pressure fluctuated through a range of 5 mm. during this period.

OBSERVATIONS ON NORMAL RATS. *Experiments with iodo-adrenochrome.* The intraperitoneal injection of 10 mgm. of iodo-adrenochrome in 0.5 cc. of olive oil into 4 normal rats, repeated on 4 successive days, scarcely affected the blood pressure (Curve 2, figure 1). Intramuscular injection of 50 and 100 mgm. of the same substance in olive oil into 2 pairs of normal rats, repeated on 4 and 3 successive days respectively, likewise left the blood pressure unaltered and did not produce any toxic symptoms (Curves 4 and 5, fig. 2).

OBSERVATIONS ON HYPERTENSIVE RATS. *Effects of iodo-adrenochrome.*[5] Five hypertensive rats were treated on 4 successive days with intramuscular injections of 5 mgm. of iodo-adrenochrome in 0.5 cc. propylene glycol. Their blood pressure dropped 15 mm. on the average during this treatment and returned to its original plateau in about as many days (Curve 8, fig. 2).

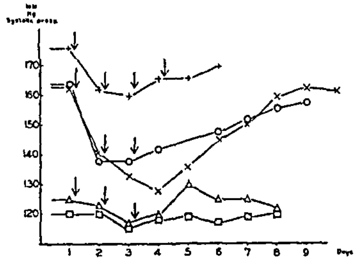

FIG. 2 EFFECT OF INTRAMUSCULAR INJECTION OF IODO-ADRENOCHROME ON HYPERTENSIVE AND NORMAL RATS

Curve 4. △—△, 50 mgm p d. in normal rats (2 animals)
Curve 5 □—□, 100 mgm in normal rats (2)

The arrows indicate days of injection

Three other hypertensive rats received intraperitoneally 10 mgm. of iodo-adrenochrome in oil on 4 successive days with a resulting maximal drop in blood pressure of 75, 65, and 60 mm. In a fourth rat 2 similar injections produced a drop of 35 mm With the exception of 1 rat, the blood pressure returned to hypertensive levels within a week after discontinuance of the injections. A composite graph of these 4 rats is given in Curve 3, figure 1.

Three hypertensive rats received 3 times 10 mgm. of iodo-adrenochrome in oil suspension each by intramuscular injection, the maximal drop was 40, 35, and 30 mm. and the gradual return to their plateau is illustrated in Curve 6, figure 2.

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[5] No difference was observed between iodo-adrenochrome prepared from levorotatory and from racemic epinephrine

Twelve hypertensive rats received intramuscular injections of 10 mgm. of iodo-adrenochrome in propylene glycol each on successive days. The injections were discontinued individually after a drop of about 40 mm. had been observed. This occurred after a single injection in 2 rats (−45, −40 mm.), after 3 injections in 4 rats (−40, −40, −35, and −30 mm., see composite Curve 7 in figure 2), after 4 injections in 4 more rats (−40, −40, −30, and −25 mm.) and after 5 injections in 2 rats (−60, and −40 mm.). With a single exception all animals returned gradually to their original hypertensive level or a few points above during a week after cessation of treatment as illustrated in the "three-injection" group.

The individual records of a group of 8 hypertensive rats which received 100–120 mgm. of iodo-adrenochrome by intramuscular injection in propylene glycol over a period of 6 to 8 successive days are given in Table 1. Under this intensive course of injections the blood pressure drop was sustained for prolonged periods in all animals except no. 4. The maximum antipressor effect was reached before cessation of treatment with the possible exception of rat no. 2. At the end of the third week, *i.e.* two weeks after the last injection, the blood pressure was still near its lowest level in this rat, but it had returned half way in three animals and had reached its original high level in all other animals; among these, three had showed abrupt rises of 35 to 40 mm. Hg from one day to another 5–10 days after the last injection.

Effect of 2-bromo-adrenochrome. Four intraperitoneal infections of 10 mgm. each of the bromo-derivative in oil suspension depressed the blood pressure from 165 to 120 mm. in 1 rat, and from 180 to 130 in another. After the injections were stopped, the blood pressure rose slowly and reached the original level after 6 and 8 days.

Effect of 1-methyl-2-iodo-3,5,6-triketo-tetrahydrindole. Four intraperitoneal injections of 10 mgm. each of a suspension of this substance in olive oil did not consistently lower the blood pressure in 2 hypertensive animals. Two intramuscular injections of 20 mgm. of a different batch, dissolved in propylene glycol, lowered the blood pressure in 4 rats by 40, 30, 30, and 15 mm.

Preparations of adrenochrome derivatives whose appearance indicated over-oxidation or polymerization and which had become insoluble in propylene glycol, left the blood pressure of hypertensive rats unchanged when injected in the same quantity and manner. This observation further confirms the specificity of the adrenochrome effect.

No correlation existed between the slight fluctuations of the animals' rectal temperature and the antipressor effect of any adrenochrome derivative. The animals showed no tendency to lose weight during the experiments.

Preliminary tests with oral administration on six successive days of 50 mgm. portions of iodo-adrenochrome, mixed with a diet limited to 4 grams of ground rat cakes per day per rat, failed to influence the blood pressure in six hypertensive rats.

A total of over 5,000 mgm. of iodo-adrenochrome and smaller amounts of the other derivatives were administered to over 60 rats in 300 individual experiments.

The above experiments in normotensive and hypertensive rats demonstrate that iodo and bromo adrenochrome fulfill for this species the requirements postulated for an antipressor substance They lower the blood pressure of the hypertensive animals over a period of days The blood pressure returns gradually to its previous hypertensive level after the injections are stopped, which proves that the nephrogenic hypertensive mechanism still persists The tested substances have no influence upon the blood pressure of normotensive animals They do not produce fever nor are they toxic in doses even ten times larger than those necessary to lower the blood pressure of the hypertensive animals

TABLE 1

Individual blood pressure records of 8 hypertensive rats during and after treatment by intramuscular injection of iodo adrenochrome in propylene glycol

DAY	RAT NO							
	1	2	3	4	5	6	7	8
	Weight							
	370 grams	310 grams	290 grams	320 grams	250 grams	310 grams	280 grams	330 grams
	mm Hg systolic blood pressure							
1	220	200	200	190	185	175	170	170
2	205	160	185	170	175	150	115	140
3	180	155	170	160	160	140	120	130
4	175	150	165	200	165	140	130	130
5	175	150	160	180	150	125	130	135
6	170	155	150	190	150	120	135	130
7	170	150	155	190	150	125	130	130
8	160	160	145	200	135	120	140	130
10	170	155	155	200	130	120	135	130
11	170	150	150		130	120	130	135
12	170	150	145		130	125	125	130
13	170	150	180		130	125	130	130
14	165	150	175		170	130	130	130
15	165	150	175		175	130	135	130
17	165		170		170	155	130	170
18	170	135	180		180	155	140	175
19	165	140	180		180	145	140	180
20	190	140	200		180	140	140	190
21	190	140	200		180	150	140	180

The vertical lines right next to the blood pressure data indicate the period during which daily injections of 20 mgm were given following the blood pressure measurement The second vertical line to the right includes the period when this treatment was continued with a daily dose of 10 mgm , whereafter administration of iodo adrenochrome was discontinued

Quite recently, similar observations, based on the same conception of pressor amine destruction, have been reported with certain synthetic paraquinones (11)

The following experiments were performed in order to elucidate the mechanism of these effects.

Atropine. One-half mgm. of atropine sulfate was injected intramuscularly together with 10 mgm. of iodo-adrenochrome per day per rat into five hypertensive rats on four successive days. The lowering effect on the blood pressure was of the same size as in the experiments without atropine. The same amount of atropine without iodo-adrenochrome had no effect on the blood pressure of the hypertensive animals.

Thus the attempt to block the parasympathetic receptor mechanism by atropine failed to suppress the antipressor action of iodo-adrenochrome.

ACUTE TESTS AND IN VITRO EXPERIMENTS. *Acute experiments with iodo-adrenochrome.* Three normal cats and six normal rabbits were anesthetized by intraperitoneal injection of 30 mgm. of nembutal per kilo of body weight and the blood pressure was recorded from a femoral artery. Intravenous injection of 50–100 mgm. of iodo-adrenochrome, either in a fine suspension in physiological saline or in a propylene glycol-saline mixture, did not lower the blood pressure of these animals. Similarly a hypertensive "Goldblatt" dog with a blood pressure plateau of 180 mm. Hg did not respond to the injection of 200 mgm. of iodo-adrenochrome under the same experimental conditions.

These observations indicate that iodo-adrenochrome has no acute depressor effect due to either dilatation of the vessel walls or inhibition of choline esterase.[6]

The cats and rabbits used in the above tests received, within 10–30 minutes after the injection of iodo-adrenochrome, 0.5 mgm. of tyramine hydrochloride or 0.1 mgm. epinephrine in 1.0 cc. of saline by the intravenous route. The response to these pressor amines was the same as that before the injection of iodo-adrenochrome. This permits the conclusion that iodo-adrenochrome does not exert a sympatholytic effect such as that of ergotamine or the dioxane derivatives of Fourneau, nor does it produce a Dale reversal for epinephrine.

Continuous infusion of tyramine hydrochloride. 1:10,000 in saline at a rate of about 40 drops per minute caused an increase in the blood pressure of two cats to a plateau about 50 mm. Hg above the initial level. A few minutes after the plateau was reached, three repeated injections of 20 mgm. each of iodo-adrenochrome failed to lower the elevated blood pressure.

The above results make it unlikely that the antipressor effect of iodo-adrenochrome is mediated by the autonomic nervous system or by direct action upon the smooth muscles of the vessel walls. The adrenochromes seem to interfere with the effectiveness of whatever pressor substance is operative in the experimental hypertension of the rat.

SUMMARY

Adrenochrome derivatives when administered parenterally lower the blood pressure of experimentally hypertensive rats but do not affect the blood pressure of normal animals. No toxic manifestations or fever are observed. The

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[6] Other oxidation products of epinephrine have been described as inhibitors of choline esterase (12).

halogeno adrenochromes fulfill for the rat the requirements postulated for an antipressor drug

Acute experiments eliminate as explanation of the observed effects a parasympathomimetic, direct muscular or sympatholytic mechanism

The catabolism of pressor amines is discussed and a chemical mechanism for the antipressor effect of quinones is suggested with due regard to species differences

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STUDIES ON THE DETOXICATION OF ORGANIC ARSENICAL COMPOUNDS

I. DETOXICATION BY MEANS OF p-AMINOBENZOIC ACID OF CERTAIN PENTAVALENT ARSENICAL DRUGS GIVEN IN MASSIVE DOSES TO RATS

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In this laboratory we have known for several years that Carbarsone (p-carbamino-phenyl arsonic acid), a drug that now enjoys extensive use in the treatment of amebiasis, possesses trypanocidal properties of a distinctly high order. With a single oral dose of this compound it is usually possible to effect a radical cure of the acute infection of rats and mice with *Trypanosoma equiperdum* and also to cure rabbits, in which animals the infection runs a more chronic, syphilis-simulating course. Occasional deaths among animals receiving average sublethal doses of Carbarsone or related compounds has stimulated interest in some of the factors that control the toxic action of arsenicals and, as a corollary, in the mechanism of trypanocidal action.

In this connection, our attention was turned to the general thesis elaborated by Fildes (1) and his associates (2, 3) to account for the bacteriostatic action of sulfonamide drugs on the basis of their specific inhibition by the structurally similar compound, *p*-aminobenzoic acid. As shown by Hirsch (4) *p*-aminobenzoic acid also inhibits the bacteriostatic action of "Atoxyl" (*p*-aminophenyl arsonic acid) *in vitro*. Extending the phenomenon into the field of parasitic protozoa, it was demonstrated by Maier and Riley (5) and by Marshall *et al* (6) that *p*-aminobenzoic acid inhibits the action of those sulfonamides that are active against the malarial parasites, *Plasmodium lophurae* in the chicken and *P. gallinaceum* in the duck.

In view of the structural relationship between "Atoxyl", "Tryparsamide" and Carbarsone, on the one hand, and sulfanilamide, on the other, it was deemed of interest to determine whether a similar mechanism underlies the susceptibility of pathogenic trypanosomes to the aforementioned pentavalent arsenical compounds. Accordingly, a trial experiment was set up in which small groups of rats infected with *T equiperdum* were treated as follows:

1. daily i.-v. doses of 1000 mgm./kg. of *p*-aminobenzoic acid[1]
2. daily i.-v. doses of 1000 mgm./kg of *p*-aminobenzoic acid plus a single small subcurative dose of Carbarsone,
3. daily doses of *p*-aminobenzoic acid plus a high dose of Carbarsone (500 mgm./kg i.-v.).

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[1] In choosing this dose we were guided by the report of Scott *et al.* (7) of these laboratories on the tolerance limits of various animals for *p*-aminobenzoic acid.

The purpose of the high dose of *p*-aminobenzoic acid and its daily administration was to avoid any minimal or evanescent inhibitory effect that might be masked by the rapid excretion of *p*-aminobenzoic acid in the presence of a more slowly excreted arsenical. If *p*-aminobenzoic acid were able to inhibit the trypanocidal action of Carbarsone, it was hoped that blood examinations at frequent intervals would reveal this effect when the concomitant dose of Carbarsone was high, even though the rats might later die from arsenic poisoning.

The result of our preliminary experiment was interpreted as showing:

(a) that *p*-aminobenzoic acid *per se* was not trypanocidal (all the rats died of trypanosomiasis about the same time as did the untreated controls);

(b) *p*-aminobenzoic acid in a high dose did not inhibit the trypanocidal action of a subcurative dose of Carbarsone (trypanosomes disappeared from the peripheral blood of the rats but reappeared a few days later and killed the animals);

(c) with the high dose of Carbarsone, again *p*-aminobenzoic acid showed no interference with trypanocidal action (several of the rats were completely cured).

To us the most interesting and, at the same time, the least anticipated outcome of this experiment was the observation that, in contrast with previous extensive experience with the intravenous administration of Carbarsone in high doses, none of the rats that received 500 mgm./kg. of Carbarsone in conjunction with *p*-aminobenzoic acid died before the fifteenth day, while most of them survived the full thirty-day observation period.

To rule out the possibility of a technical error, e.g., in dosage, as well as to determine whether the high survival rate was to be ascribed more properly to the vagaries of statistically non-significant chance rather than to a genuine detoxication of Carbarsone by *p*-aminobenzoic acid, the experiment was repeated. When the results of the first trial were essentially duplicated, more experiments were designed to explore the range of the detoxicating action of *p*-aminobenzoic acid not only with Carbarsone but also with other arsenical drugs.

The present report[2] is restricted to some of the well known substituted phenyl arsonates, leaving a consideration of the rather different situations that obtains with respect to certain trivalent arsenicals for a later publication.

EXPERIMENTAL PROCEDURE. Solely to facilitate calculation of dosage in rat protection experiments, preference was given to the use of animals weighing approximately 100 grams. Otherwise no selection as to sex or age was made from our stocks of heterogeneous races of rats secured from several dealers. With the depletion of stocks, we have often used rats weighing from 80 to nearly 200 grams without detecting any essential difference in the protection afforded by *p*-aminobenzoic acid. All rats, including the controls essential to each experiment, were maintained on a regular diet of well balanced commercial "Chow" in large communal cages. For parenteral as well as oral administration, the various sparingly soluble phenyl arsonic acids and *p*-amino-benzoic acid (C. P. quality) were made up daily into 5 to 20% aqueous solution by adding the necessary quantity of N/10 alkali. Since unprotected animals rarely lived beyond the seventh day while protected animals lived indefinitely, tests were usually brought to a conclusion from 10 to 14 days after the administration of the arsenical drug.

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[2] A preliminary report of these findings was made in "Science," January, 1943.

The results of some typical detoxication experiments are collated and summarized in table 1

TABLE 1

Detoxication of pentavalent arsenical compounds by p aminobenzoic acid

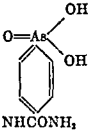
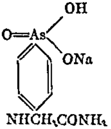
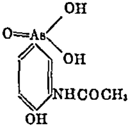
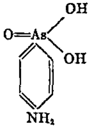
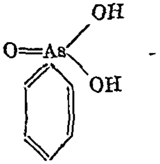
DRUG			*p*-AMINOBENZOIC ACID		NUMBER OF RATS*		PERCENTAGE SURVIVAL	
Formula	Dose	Mode of administration	Dose	Mode of administration	Used	Survived	With *p* aminobenzoic acid	Controls
	mgm / kg		mgm /kg					
Carbarsone  	500	i m	2000 × 4	i -p	24	22	91	
	500	i m	controls		24	4		16
	750	i v	1000 × 6	oral	10	10	100	
	750	i -v	controls		7	1		14
	1000	i v	1000 × 3	i p	20	18	90	
	1000	i v	controls		31	4		13
	1500	i -p	3000 × 3	oral	15	13	87	
	1500	i p	controls		45	0		0
"Tryparsamide"  	2500	sub cut	2000 × 3	oral	10	10	100	
	2500	sub cut	controls		10	6		60
	3000	oral	1000 × 3	i -p	10	8	80	
	3000	oral	controls		10	3		30
	3200	i p	1000 × 3	i -p	16	14	87	
	3200	i p	controls		26	5		19
Acetarsone  	4000	oral	2000 × 3	i p	10	9	90	
	4000	oral	controls		10	6		60
	400	i p & i m	2000 × 3	oral & i -p	40	35	87	
	400	i p & i m	controls		20	0		0
Arsanilic acid  	250	i p	2000 × 4	i p	10	10	100	
	250	i p	controls		10	5		50
	350	i p	2000 × 2	i -p	10	10	100	
	350	i p	controls		9	5		55
	400	i p	2000 × 3	i p	10	10	100	
	400	i p	controls		22	2		9

TABLE 1—*Continued*

DRUG			*p*-AMINOBENZOIC ACID		NUMBER OF RATS*		PERCENTAGE SURVIVAL	
Formula	Dose	Mode of administration	Dose	Mode of administration	Used	Survived	With *p*-amino benzoic acid	Controls
	mgm./kg.		*mgm./kg.*					
Phenyl arsonic acid	50	i.-p.	3000 × 3	i.-p.	14	13	92	
	50	i.-p.	controls		14	2		14
	60	i.-p.	3000 × 3	i.-p.	10	10	100	
	60	i.-p.	controls		10	0		0
	70	i.-p.	3000 × 3	i.-p.	10	10	100	
	70	i.-p.	controls		10	0		0

* Some of the results presented here are aggregates of several smaller experiments conducted at various times with the arsenical dosage specified.

Discussion. So large has been the number of animals used in the toxicity experiments summarized in the foregoing table, and so high is the difference in the survival rate among rats that have received p-aminobenzoic acid, that it is unnecessary to seek the aid of statistical analysis to establish, beyond question, the protective rôle played by p-aminobenzoic acid. Critically measured, as it has been in these experiments, by the extreme criteria of death or indefinite survival, following a single administration of the arsenical, the detoxicating properties of p-aminobenzoic acid will be recognized as being of an order much superior to that of certain detoxicants of other toxic principles where the order of protection is measured by a mere prolongation of the experimental animal's life for a few days beyond that of the controls, or where protection can be demonstrated only by a comparison of the extent of histological damage in treated and control animals.

The protective action of p-aminobenzoic acid can be detected in the majority of rats within 24 hours of administration. While death may sometimes supervene earlier, the toxic effects of pentavalent arsenicals (in contrast to trivalent arsenicals) are, as a rule, of the delayed type. The onset of toxic symptoms is marked, usually, with a diarrhea which may become sanguineous. Symptoms referable to the effect of the drug on the central nervous system commence to develop in about 24 hours and take the form of torticollis, head tic, tremors and gyrations (so-called "waltzing"). The rat goes off feed; if it does not die earlier, by the third day emaciation with matting of the eyelids and whiskers by the coproporphyrin-containing excretion from the Harderian glands (8) becomes evident. A flaccid paralysis of the hind quarters is often the harbinger of death. Some rats, however, miraculously recover when they appear moribund. Signs of arsenic poisoning may appear in p-aminobenzoic acid treated rats, but the majority either show no signs of poisoning or the stigmata are minimal and transient.

Extended consideration of the dosage of *p*-aminobenzoic acid is reserved for treatment in a subsequent paper in this series, but a few remarks at this point may indicate the reason for the high dosage used and multiple treatments. In this connection we may emphasize the fact that the arsenic detoxicating capacity of *p*-aminobenzoic acid is an incidental discovery and much remains to be learned about it. Because of the notorious variability in the tolerance of individual rats to arsenical poisoning, it is almost as difficult to establish the minimum LD_{100} as it is to determine the minimum LD_{50} without undertaking experiments on a large number of animals. For this reason it has not been deemed desirable at the present juncture to determine accurately the number of lethal doses (or LD_{50}'s) of the various pentavalent arsenicals against which *p*-aminobenzoic acid affords complete protection. Since it is reported (9) that *p*-aminobenzoic acid, like benzoic acid, (10) is rapidly absorbed by mouth and almost entirely excreted by way of the kidneys within 24 hours, and since it is generally believed that the pentavalent arsenicals are rather slowly and only partially eliminated (11) so that some of the symptoms of poisoning are attributed to a cumulative action of unexcreted arsenic, it was considered necessary to establish a high *p*-aminobenzoic acid concentration in the blood and to maintain it until all danger had passed. Work still in progress in our laboratory proves conclusively that in nearly all of our previous experiments (summarized in the preceding table) more *p*-aminobenzoic acid was administered than was necessary to protect the majority of rats. In fact, the dosage in certain instances was so high as to approach the limit of *p*-aminobenzoic acid tolerance and perhaps even to account for the death of a few rats that might otherwise have survived.

Finally, it may be of interest to note that, aside from the efforts of organic chemists since the time of Ehrlich to introduce a variety of side chains into the benzene ring of *p*-aminophenylarsonic or arsanilic acid (so-called "Atoxyl") with the main purpose of reducing the high toxicity of this basic compound, no effective method is recorded of lessening the hazard of medication with the several pentavalent arsenicals now in clinical use. This is in contrast with the apparently successful attempts that have been made to reduce toxicity, both acute and chronic, of the trivalent arsenicals. Thus, Voegtlin, Dyer and Leonard (12) have shown that "arsenoxide" (*m*-amino *p*-hydroxy phenyl arsenoxide) in otherwise lethal quantities could be safely administered to rats when accompanied or preceded by an injection of reduced glutathione or certain other sulphydryl-rich compounds, while Durel (13) demonstrated the value of ascorbic acid in reducing the toxicity of "novarsenobenzol" (Neoarsphenamine) in mice. In addition there are now many clinical reports of the value of ascorbic acid, liver extracts, etc., in counteracting an induced sensitivity or in preventing the toxic side actions in patients under treatment with Neoarsphenamine.

With reference to the detoxicating effect of *p*-aminobenzoic acid against the pentavalent arsenicals, it may be recalled that Fildes (*loc. cit.*) made the interesting suggestion that *p*-aminobenzoic acid, in addition to inhibiting the bacteriostatic action of the sulfonamides, might be expected to counteract the toxic effects that sometimes accompany medication with sulfanilamide. No support, however, has been found for this conjecture in the clinical studies of Strauss and Finland (14). Further work that we hope soon to publish shows no direct relationship between the chemical structure of the particular phenyl arsonates and the detoxicating agent.

SUMMARY

1. *p*-aminobenzoic acid has been found highly effective in reducing fatalities among rats to which high, acutely poisonous doses of Carbarsone, Acetarsone, "Tryparsamide", arsanilic acid, and phenyl arsonic acid have been administered. No close structural similarity exists between these several phenyl arsonates and *p*-aminobenzoic acid.

2. Detoxication is independent of the route whereby either the phenyl arsonic compound or the *p*-aminobenzoic acid is given.

3. No inhibition by *p*-aminobenzoic acid of the trypanocidal action of Carbarsone, "Tryparsamide" or arsanilic acid (= "Atoxyl") was detected in rats infected with *Trypanosoma equiperdum*.

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ANESTHESIA

VIII. The Anesthetic Action of Isopropenyl Methyl Ether[1]

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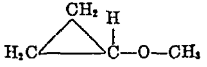
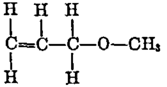
In former communications (1 and 2) the authors reported their studies on the anesthetic properties of cyclopropyl methyl ether (Cyprome Ether) and its isomer allyl methyl ether. The former gave promise in its pharmacologic investigation and has been used successfully in man (3). The latter was irritating and hepatotoxic to several species of laboratory animals and was hence not used clinically. In the study of the isomers of cyprome ether the authors prepared one of its isomers in which the double bond is attached to the carbon atom united with the oxygen atom of the ether linkage. The structural relationships are apparent from the following formulas.

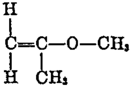


Cyprome Ether



$\mathrm{H_2C{=}CH{-}CH_2{-}O{-}CH_3}$

Allyl Methyl Ether



$\mathrm{H_2C{=}C(CH_3){-}O{-}CH_3}$

Isopropenyl Methyl Ether

Isopropenyl methyl ether is a volatile, colorless liquid with a characteristic ethereal odor; it boils at 35°C. and has a specific gravity of 0.763 at 25°C.

Anesthesia in the monkey. Five large Rhesus monkeys were each anesthetized twice with isopropenyl methyl ether. The technic is described in detail in our previous communication (1). The induction period was short; frequently the animals passed into the first plane of surgical anesthesia without struggle. During induction, salivation was not marked and the bronchial tree remained quite free from mucus throughout the anesthesia. Surgical anesthesia was uneventful. Breathing was often stertorous but deep and regular. Relaxation of the musculature of the abdomen and extremities was complete under surgical anesthesia. There were no incoördinated leg movements during anesthesia. Pain reflexes were abolished Recovery from anesthesias of 15 minutes duration was very prompt, resembling the recovery from divinyl oxide anesthesias. There was little excitation during the recovery period. The quantities of isopropenyl methyl ether required were approximately two-thirds of the amounts of ethyl ether required for similar anesthetic syndromes.

Anesthetic index (dog). The general plan of administering the anesthetic was

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[1] The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Co. of Cleveland, Ohio

the same as that described previously (1). However, when isopropenyl methyl ether was administered at the rate of 0.5 cc. per minute the animal frequently regained consciousness during the intervals between doses. With this agent we adopted the plan of administering 2 cc. of the agent at the beginning of induction and 1 cc. per minute to respiratory failure. Three dogs were used to determine the anesthetic index of ether using the larger dosage. The values appeared to be approximately 20% higher than when the dosage of 0.5 cc. per minute was used. All animals recovered when artificial respiration was instituted. Making allowances for density differences isopropenyl methyl ether appears to be approximately twice as potent as ethyl ether in the dog. The difference between the induction dose and the respiratory arrest dose of ethyl ether is 1 cc. per kgm.; with isopropenyl ether it is 2 cc. per kgm. The data are shown in table 1.

Blood pressure studies (dog). The effect of isopropenyl methyl ether on the blood pressure was determined by cannulating the femoral artery under procaine anesthesia and preparing the animal for a blood pressure tracing in the usual manner. The respiratory tracings were made by means of a chest tambour. After a normal tracing, isopropenyl methyl ether was administered by the same technic employed to measure the anesthetic index. In four experiments respiratory failure occurred before cardiac stoppage. The blood pressure was depressed slightly at the plane of surgical anesthesia and deeply at the point of threatened collapse.

TABLE 1

Anesthetic index

DOG NUMBER	SEX	WEIGHT	INDUCTION	RESPIRATORY FAILURE	ANESTHETIC INDEX
		kgm	*cc./kgm*	*cc./kgm.*	
1	F	8.5	0 47	2.47	5.26
2	F	10.5	0.48	2.57	5.36
3	M	10.4	0.48	2.98	6.21
4	M	8.4	0.48	2.14	4.46
5	M	11.5	0.44	1.83	4.16
6	F	7.0	0 43	2.00	4.65
7	F	5.8	0.52	2.41	4.63
8	M	8.3	0.60	3.50	5 83
Mean			0.49	2.49	5.07

Electrocardiographic studies (monkey). Three Rhesus monkeys were anesthetized twice with isopropenyl methyl ether and were kept at the surgical level for 10 minutes. Just prior to anesthesia, and during surgical anesthesia, electrocardiograms were made. During the six anesthesias neither animal revealed any significant differences in the rate, form or regularity of the E.C.G.

Effect on perfused heart (frog). Isopropenyl methyl ether was dissolved in Howell-Ringer's solution and perfused through the frog's heart *in situ*. The dilution which demonstrated a liminal effect within one minute was determined to be approximately 0 007 molar A concentration of 0 014 molar (100 mgm %) was perfused repeatedly for long periods of time In 7 animals 0 014 molar isopropenyl methyl ether produced slight diminution in rate and amplitude of beat as a typical effect Occasionally after long perfusion an extra systole was observed and the refractory period was prolonged Ethyl ether in concentrations of 0 014 molar caused similar effects in these experiments

Liver function tests (monkey) Three Rhesus monkeys were subjected to the bromsulphthalein liver function test as set forth in former studies (4) Twenty four hours after a 30 minute anesthesia with ispropenyl methyl ether the dye excreted, in all cases, was not significantly different from the preanesthetic excretion rate

Blood chemistry studies (monkey) Three Rhesus monkeys were anesthetized to the surgical plane of anesthesia for 15 minutes with isopropenyl methyl ether Prior to anesthesia and 24 hours later blood samples were drawn for analysis No significant changes were observed in carbon dioxide combining power or urea nitrogen

TABLE 2

Induction concentration in mice

PARTIAL PRESSURE	CC PER LITER	NUMBER OF MICE PER JAR	NUMBER OF MICE USED	PER CENT ANESTHETIZED
%				
2	0 08	5	10	0
2 5	0 10	5	10	0
3	0 12	5	10	0
4	0 16	5	35	91

Concentration required for anesthesia (mouse) The concentration of isopropenyl methyl ether required to induce anesthesia was determined by typical partial pressure experiments described previously (1) The results are shown in table 2 With ethyl ether, in our previous studies, 4% partial pressure produced no anesthesias and 5% anesthetized 40% of the animals

Delayed anesthetic deaths (rat) Twenty male adult rats were anesthetized with isopropenyl methyl ether to the surgical plane and maintained in this condition for one half hour This was repeated every 4 days on 5 animals for 3 weeks One of the 20 animals died 4 days after anesthesia and one animal of the group died after 4 anesthesias

Histological studies of viscera (rat, dog, monkey) Twenty of the rats used in the delayed anesthetic death experiments were sacrificed for histological study Their livers and kidneys were found to be free from significant changes Three dogs were anesthetized lightly with cyclopropane and liver biopsies performed The anesthetic agent was changed to isopropenyl methyl ether and maintained at the surgical level for one hour and a second hepatic biopsy performed There were no significant pathological findings Three Rhesus monkeys were subjected to the foregoing biopsy procedure as performed on the dogs The findings in this series coincide with those described with the dog anesthesias

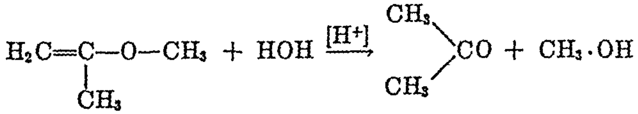
Clotting time and hemolysis (monkey). The clotting time of blood was determined in 2 normal Rhesus monkeys by the capillary tube method. The average clotting time was approximately one minute. Within the error of the experiment this period was neither diminished nor increased under surgical anesthesia with isopropenyl methyl ether.

Ten cc. volumes of isopropenyl methyl ether of varying concentrations in normal salt solution were maintained at 37°C. To each of these was added 0.1 cc. of defibrinated dog's blood and the time required for hemolysis was observed. After 4 hours at 37°C. concentrations of 100 mgm. % produced no hemolysis. At room temperature concentrations were studied up to 200 mgm. % for a period of 5 hours. During this period no hemolysis was observed.

Preanesthetic medication (monkey and dog). With monkeys and dogs morphine-atropine medication influenced the anesthesia with isopropenyl methyl ether in the same manner in which it affects the anesthesia of ethyl ether. In monkeys inducing isopropenyl methyl ether anesthesia with nitrous oxide-oxygen or cyclopropane-oxygen mixtures was uneventful. Preanesthetic medications with pentobarbital sodium was found to be entirely compatible with isopropenyl methyl ether anesthesia. Eight experiments in all were conducted.

Physical properties. Solubility in water. Four cc. of isopropenyl methyl ether was mechanically agitated with 100 cc. of water for 2 hours at 25°C. in a "Cassia Flask." The two liquids were allowed to separate for 12 hours and the volume of the supernatant isopropenyl methyl ether was measured. The solubility of isopropenyl methyl ether was found to be 1.2 cc. in 100 cc. of water.

Hydrolysis studies. It is well established that α-unsaturated ethers do not enjoy the stability to hydrolysis that is characteristic of saturated ethers. Isopropenyl methyl ether was observed to be rapidly hydrolyzed in water acidified by strongly dissociated acids according to the following equation:



$$H_2C{=}\underset{\displaystyle CH_3}{\underset{|}{C}}{-}O{-}CH_3 + HOH \xrightarrow{[H^+]} \begin{matrix}CH_3\diagdown\\ CH_3\diagup\end{matrix}CO + CH_3{\cdot}OH$$

In neutral water this hydrolysis occurred slowly. This prompted us to study the hydrolytic rate in phosphate buffer pH 7.4. In this solution more than 3 grams of the anesthetic hydrolyzed within 1 hour by agitation with 100 cc. of the buffer solution at 25°C. The fate of the anesthetic in the blood accordingly became a matter of serious consideration.

Fate in blood (monkey). As isopropenyl methyl ether is almost instantly converted into acetone and methanol by contact with strongly acidic solutions, the determination of acetone in the blood in the presence of the undecomposed anesthetic required a careful procedure. Monkeys were anesthetized and the freshly drawn blood was hemolyzed by the addition of water containing alkali. This was distilled into neutral permanganate solution which oxidized the unhydrolyzed anesthetic but not the acetone. The acetone was distilled and determined colorimetrically by the method of Behre and Benedict (5). The method was found to be reliable on fresh blood samples containing known amounts of isopropenyl methyl ether and acetone.

During short anesthesias (15 to 30 minutes) with isopropenyl methyl ether the blood of the monkey contained from 15 to 30 mgm. % acetone and after a 2 to 3 hours anesthesia this concentration increased to 150 mgm. % with presumably an equimolar concentration of methanol One monkey survived a 3½ hour anesthesia with isopropenyl methyl ether with apparently no acute or delayed harmful effects.

In the dog, frequently we were unable to produce respiratory arrest with isopropenyl methyl ether. This observation was the first indication of the decomposition of the anesthetic agent and this suspicion was later confirmed in buffer solutions and monkey's blood

SUMMARY AND CONCLUSIONS

Isopropenyl methyl ether is an anesthetic agent in many species of laboratory animals. When measured by many of the criteria for determining the desirable properties of an inhalation anesthetic, isopropenyl methyl ether showed great promise of clinical usefulness Owing to the decomposition of the ether in the circulation with the formation of methanol, and the insidious and capricious action of this compound on the eye, we believe that isopropenyl methyl ether is unavailable in man as a safe anesthetic agent

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STUDIES ON ANTIMALARIALS

The Accumulation and Excretion of Atabrine[1]

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The widespread use of atabrine points clearly to the need for a more thorough study of its pharmacologic and toxicologic behavior. Quantitative data concerning the rate and extent of accumulation of atabrine in experimental animals after repeated dosage, and the influence of diet and disease on the detoxication rate, are essential to the proper use of the compound in the chemotherapy of malaria.

Atabrine is generally considered to be a cumulative drug though the evidence for this is rather indirect. The low blood concentrations (1–5) together with the small amounts found in the urine and feces (2–4, 6–12) indicate that atabrine is rapidly absorbed and taken up from the blood by the tissues. The atabrine content of tissues has been studied (5, 13) after a single relatively large dose but the accumulation in the tissues after long continued administration of small doses has not been investigated. The slow fall in concentration of atabrine in the tissues (3) together with the observation that it is still detectable in the urine and feces 69 or 70 days after the cessation of treatment (11) suggests that if it were given on a long term basis, accumulation would occur. Hecht (6) and Martin, Cominole and Clark (14) observed non-characteristic pathological changes in animals following large oral doses of atabrine. With small doses, observable cumulative effects are absent or only slight over a period of 6 to 8 weeks (14).

The yellow pigmentation frequently seen after atabrine administration has been considered the most important clinical evidence of accumulation. It appears early in the course of treatment and may persist for as long as eighteen weeks after cessation of treatment (15). In animal experiments this pigment has been shown to be fluorescent (6) and is undoubtedly either atabrine or a derivative. Other symptoms suggestive of accumulation are headache, mental depression and psychoses.

The general acceptance of progressive accumulation in the tissues is probably responsible for such recommendations as those of Dawson, Gingrich and Hollar (16), "It seems reasonable that as a general rule, to lessen the risk of toxic effects from cumulation, atabrine treatment should not be repeated within eight weeks of a previous course, and that the drug should be dispensed only on prescription of a physician "

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[2] These studies were submitted to the University of Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy, December, 1942.

[3] John J. Abel Fellow in Pharmacology.

Our studies were undertaken with a view to determining in dogs the amount of atabrine excreted in the urine and feces and the amount of accumulation in the tissues after small daily oral doses, comparable to those used in the suppressive treatment of malaria The accumulation in the tissues was also determined after large daily oral doses and after simultaneous administration of atabrine and sulfonamide drugs

METHODS In all experiments the dogs were fed at the same time each day and atabrine dihydrochloride given orally one half hour later Dosage levels of 5 and 50 mgm /kgm were used Unpublished work in this laboratory has shown that doses of 50 mgm /kgm induce vomiting in dogs in 70% of the cases when given in gelatin capsules and in 96% of the cases when given as a water solution by stomach tube To avoid this undesirable reaction the drug was given in the form of tablets coated with cellulose acetate phthalate, a new enteric coating supplied by Eastman Kodak Company The smaller doses were given in gelatin capsules Feces and urine collections were made daily just before feeding All analyses and doses are expressed in terms of atabrine dihydrochloride

Blood level and urinary and fecal excretion studies were made on four dogs which were kept in individual metal metabolism cages, the urine and feces were collected daily just before the administration of atabrine Blood samples were taken daily just before giving atabrine during the first week, and at 24 and 48 hour intervals after the last dose of each weekly series thereafter Estimations of the concentration of atabrine in the blood of these dogs were made by direct iso amyl alcohol extraction of laked whole blood buffered to pH 8 5 and determination of the fluorescence of the alcohol extract During the first week of the experiment atabrine in the urine and feces was determined daily After this time aliquots of the daily collections were pooled and stored in the refrigerator until the end of the week when determinations of the weekly excretion were made The feces of the animals receiving 50 mgm /kgm were analyzed for atabrine to determine the absorption from the enteric coated tablets Control experiments showed no change in the atabrine content on storage in the refrigerator

The distribution in the tissues was studied in 24 dogs In all experiments the dogs were sacrificed 48 hours after the last dose Two dogs were analyzed after a single dose of 5 mgm /kgm and two each after one, two, three and four weeks courses of atabrine 5 mgm / kgm per day for six days each week Two dogs which had received approximately 30 mgm /kgm per week for eight weeks followed by 15 mgm /kgm twice each week for four and one half weeks were given 5 mgm /kgm per day for three days and then analyzed Two dogs were sacrificed after seven and two after fourteen daily doses of 50 mgm /kgm Four dogs were given 50 mgm /kgm of sulfathiazole three times a day simultaneously with the third week of atabrine (5 mgm /kgm per day, six days a week), and four dogs were given 50 mgm /kgm of sulfadiazine three times a day simultaneously with the fourth week of atabrine (5 mgm /kgm per day) Free and total blood sulfathiazole or sulfadiazine were determined on these eight dogs and also on four controls for each sulfonamide

The concentration of atabrine was determined in the adrenals bile, blood bone marrow, brain heart kidney, liver, lung, muscle, pancreas, skin and spleen Analyses were done in duplicate except for the adrenals, bile and bone marrow The deviation of the duplicates from the average was less than 5%

Method of Atabrine Analysis The sample was blended with at least four parts by weight of water, and 25 gram aliquots transferred by pipette to tared 100 cc beakers After weighing 25 cc of *2N* HCl was added and the samples digested by warming on a steam bath for two hours The volume was maintained by addition of distilled water

After the digestion period, the sample was cooled in the refrigerator and saturated NaOH added to make the mixture alkaline The sample was immediately transferred to a separatory funnel and an alkaloid extract prepared exactly as already described (17) for the determination of quinine One cc of concentrated NH_4OH was added to the extract in a 50 cc. volumetric flask and water added to the mark. For the fluorometric assay the Coleman Model 12 Electronic Photofluorometer was used with B-1 and PC-1 filters, with quinine as a reference standard.[4] This method was primarily designed for tissue and excretion studies. The concentration of atabrine in the blood is too low for accurate determination by this method except when very large doses of atabrine are used.

A dog which had never received atabrine was sacrificed, and the various tissues analyzed for "apparent atabrine" concentration. Most tissues were found to have a small amount of extractable fluorescent material. This was especially noticeable in the feces, but even here the concentration was 1 mgm./kgm. or less. This amount was considerably below the concentration observed in experiments with atabrine. The recovery of known amounts of atabrine added to tissues ranged from 92 to 113%. Recovery from aqueous solutions of pure atabrine was always within 10%.

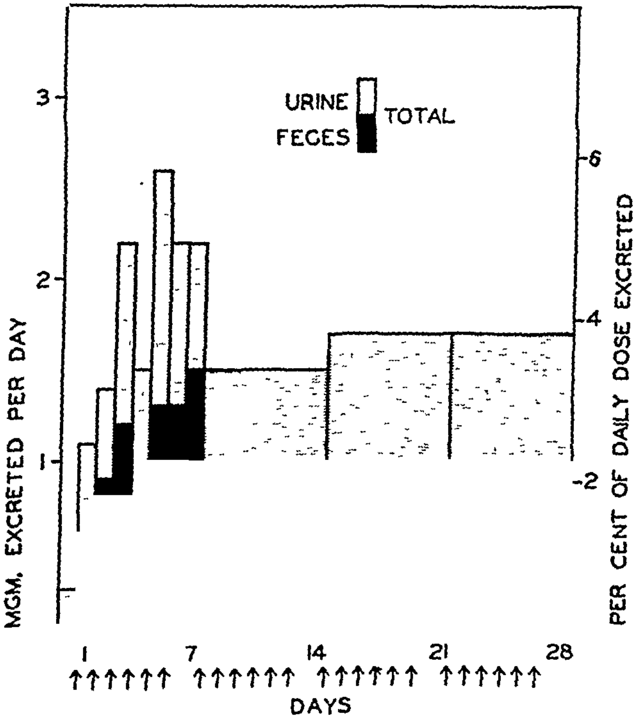

FIG. 1. EXCRETION OF ATABRINE IN THE URINE AND FECES
Arrows designate days on which atabrine was given

RESULTS. Figure 1 shows the daily excretion of atabrine in the urine and feces for one week and the average daily excretion for each of the three succeed-

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[4] Later studies on the sensitivity and specificity of this method have led to two improvements. The results are more reliable when the alkaloid extract is made up in a final concentration of 50% alcohol with 1 cc. of concentrated NH_4OH added for each 50 cc., and more nearly specific for atabrine when the B_2 PC-2 filter combination is used. Certain atabrine derivatives have been found to fluoresce more strongly to the 365 mm. Hg. line (B_1) than to the 436 mm line (B_2), and the resultant fluorescence of these derivatives passes the PC-1 filter more readily than the PC-2.

TABLE 1

Distribution of atabrine in tissues

TISSUE	DOSAGE LEVEL										
		50 mg./kg		5 mg./kg							
	Number of daily doses										
	0	7	14	1	6	12	18	24	78*	18†	24‡
	Concentration of atabrine dihydrochloride in mg./kg. of tissue										
Adrenals	0.1	198 63	445 199	7.3 3.3	18.3 10.7	31.2 20.1	22.5 15.6	26.7 13.3	12.7 15.4	3.1 15.4 3.9 5.6	15.8 18.9 2.1 8.2
Bile	0.1	207 23.4	310 208	1.9 3.4	9.5 22.4	2.3 7.9	2.4 10.7	8.8 14.9	6.7 10.4	1.3 3.4 2.4 10.5	6.7 2.8 1.4 2.6
Blood	0.0	1.0 0.3	1.5 2.8	0.0 0.0	0.2 0.1	0.1 0.0	0.0 0.3	0.3 0.0	0.1 0.0	0.0 0.0 0.0 0.0	0.0 0.0 0.0 0.0
Bone marrow		88.0 16.5	100 107	0.8 0.0	14.7 6.6	5.7 2.4	5.0 15.2	1.9 1.5	5.9 8.1	1.2 4.3 2.3 3.9	7.4 2.2 0.5 2.4
Brain	0.3	7.9 1.9	9.8 5.9	0.4 0.8	1.5 0.8	1.9 1.1	0.7 0.8	1.0 0.3	0.7 0.9	0.1 0.3 0.2 0.4	0.9 0.7 0.2 0.3
Heart		60 10	91 48	1.8 1.0	5.6 2.8	6.4 4.0	4.6 4.9	5.4 2.4	1.6 3.7	0.1 1.1 1.2 0.6	11.2 2.6 0.8 1.1
Kidney	0.0	49 16	90 88	1.8 1.0	7.8 2.8	5.5 4.0	3.9 4.9	11.5 2.4	5.0 3.7	1.3 3.3 2.6 1.5	11.7 10.3 1.6 2.9
Liver	0.2	737 59	995 705	8.0 5.7	28.5 33.8	40.0 29.0	21.0 25.9	17.0 10.0	12.2 35.2	2.9 12.8 5.9 4.0	19.0 25.0 6.8 6.6
Lung	0.1	77 50	166 154	2.0 2.0	16.0 8.9	13.6 14.8	11.2 11.4	26.7 8.0	13.0 19.5	3.6 0.1 6.7 2.2	22.0 15.6 8.1 7.4

* These dogs received the equivalent of 78 doses over a period of 13 weeks

† These dogs received in addition 50 mg./kg. of sulfathiazole t.i.d. the last 6 days

‡ These dogs received in addition 50 mg./kg. of sulfadiazine t.i.d. the last 7 days

TABLE 1—*Continued*

TISSUE	DOSAGE LEVEL										
		50 mg./kg.		5 mg./kg.							
	Number of daily doses										
	0	7	14	1	6	12	18	24	78*	18†	24‡
	Concentration of atabrine dihydrochloride in mg./kg. of tissue										
Muscle		13	21	0.6	3.2	1.9	0.5	1.5	0.7	0.1	5.8
		10	22	0.1	1.6	1.2	2.8	0.5	1.1	0.4	0.6
										3.5	0.1
										4.3	0.2
Pancreas	0.1	120	372	7.8	42.0	33.0	22.5	48.8	43.6	3.6	5.7
		62	242	3.7	23.5	30.0	50.0	21.8	63.5	18.1	27.2
										6.3	7.2
										15.4	19.0
Skin		16	51			1.4	0.8	5.5	4.2	1.5	2.8
		10	15	1.0	1.3	1.1	2.1	2.6	5.4	1.3	1.5
										1.0	1.6
										0.4	4.4
Spleen	0.1	168	232	5.2	42.0	22.0	11.6	32.4	21.2	2.8	22.2
		71	263	4.4	16.5	42.5	30.2	15.0	50.0	25.6	21.2
										8.4	8.4
										6.6	2.2

ing weeks. In all cases the per cent of the daily dose excreted in the urine and feces was less than six although a considerable amount of fluorescent atabrine derivatives, not extracted by this method, was present in the urine. *Stabilization* of the per cent of the daily dose excreted was attained within two weeks.

At the time of these experiments, no satisfactory method was available for the determination of low blood levels of atabrine. The fluorescence of the isoamyl alcohol extract of the laked blood gave an indication of the amount of total fluorescent alcohol-soluble material but it was not considered sufficiently sensitive or specific to warrant presentation of the individual data obtained. However, in no instance did the concentration of extracted fluorescent material (calculated as atabrine dihydrochloride) rise above 1 mgm./l., and the average level was about 0.5 mgm./l. These observations are in agreement with those of other investigators (1–5) and serve only to emphasize the rapid removal of atabrine from the blood.

In the dogs receiving 50 mgm./kgm. per day as enteric coated *tablets no* vomiting occurred. The animals showed some weight loss and a severe diarrhea, but in spite of the diarrhea, fecal analyses showed that approximately 80% of the administered drug had been absorbed. The high concentrations attained in the tissues are further evidence of the completeness of absorption.

The distribution of atabrine in the tissues is shown in table 1. Although the spread of values obtained from each pair of dogs on the 5 mgm./kgm. dosage level was wide, in general the maximal concentrations were attained in one week. The adrenals, heart and liver gave slightly higher values for the second week. No tissue showed a significantly higher concentration at 13 weeks with the exception of the skin and possibly the pancreas. The liver showed a significant drop in the two to four week period. The adrenals, kidney, bile, bone marrow, heart, muscle and brain showed equivalent or slightly lower values at thirteen weeks. The tissue concentrations attained with the 50 mgm./kgm. dose were approximately ten times those found after 5 mgm./kgm. dosage.

Since after the first or second week of daily atabrine administration, neither the elimination nor the storage of the drug is increased, and since an average of less than 4% is eliminated in the urine and feces, the dog must convert approximately 96% of the daily intake to other substances. Unpublished data from this laboratory show that there is no histologically detectable damage to the liver after eight weeks and no apparent effect on liver function (by bromsulfalein test) or the general health of the dogs after 32 weeks during which they received 30 mgm./kgm. per week.

In the dogs which received sulfathiazole simultaneously with atabrine the tissue levels attained were considerably lower than when atabrine was given alone for an equal period of time. With sulfadiazine the deviations from the controls were not so marked but the trend was in the same direction as with sulfathiazole. The atabrine had no effect on the blood levels of either free or total sulfathiazole or sulfadiazine.

SUMMARY

The excretion of atabrine in the urine and feces was studied throughout a period of four weeks of daily atabrine administration. The average daily excretion soon reached a level of less than 4% of the daily dose.

Determinations of the atabrine concentration in various tissues of the dog 48 hours after the last of a series of small daily oral doses showed the maximum concentrations in most tissues to be reached within two weeks.

Higher tissue levels are attained with larger doses, those reached with 50 mgm./kgm. being about ten times those reached with 5 mgm./kgm.

Cellulose acetate phthalate is a satisfactory enteric coating for administering relatively large doses of atabrine since no vomiting occurs and about 80% of the administered drug is absorbed.

The administration of atabrine with either sulfathiazole or sulfadiazine did not affect the blood levels of the sulfonamides, but the concentration of atabrine in the tissues was lowered.

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HEMATIC AND ORGANIC REACTIONS IN STANDARDIZED AND GRADED HISTAMINE SHOCK IN DOGS

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The development of methods for the production of a standardized and graded secondary shock in animals is of importance not only for a study of the still controversial dynamics of this condition, but is also for the reliable evaluation of the relative efficacy of therapeutic procedures intended for use in the management of shock in man. Several attempts have been made recently in this direction. Wiggers and Werle (25) tried to cause a standardized and irreversible hemorrhagic shock in dogs and found that the dangerous factor in this type of shock is not as definitely related to the percentile reduction in blood volume as it is to the degree and duration of hypotension which eventuates. Noble and Collip (16) reported a quantitative method for the production of traumatic shock without hemorrhage in unanesthetized rats and guinea pigs. When these animals were whirled in a rotating drum with two projections against which the animals were thrown, the number of rotations determined the degree of trauma obtained, i.e., the survival time and percentage of deaths. A second method for the uniform production of experimental shock by crush injury was recorded by Duncan and Blalock (3). These investigators place the hindlimb of dogs between two boards supplied on the inside with triangular strips of wood which were placed so that when the boards were approximated their coaptation resulted in a cog- or gear-like arrangement in which the muscular tissue of the thigh was crushed. When this device was applied under a definite pressure and kept on for five hours, 18 of 19 dogs died with the symptoms of shock, such as hemoconcentration, hypotension, and swelling of the crushed leg due to the escape of considerable amounts of plasma into the injured tissue.

In previous experiments with histamine shock (9) histamine was administered as a suspension in a medium consisting of oil and a lanolin base (Falba) so as to slow up the absorption of the histamine and to prolong its action, simulating thereby conditions existing in traumatic shock. It became essential for a reliable evaluation of the efficacy of therapeutic agents (10) to elaborate upon this procedure so as to obtain a standardized and graded method. The results of experiments aimed at such elaboration are presented in this communication.

EXPERIMENTAL PROCEDURE Mongrel dogs were used in the experiment. These were injected subcutaneously with histamine dihydrochloride suspended in a base of cottonseed oil (U.S P) and "Falba" Absorption Base (Pfaltz and Bauer, Inc), which is a derivative of lanolin. The vehicle used for suspending the histamine dihydrochloride was prepared by mixing three parts of the oil with one part by weight of the absorption base. The histamine suspension needed for an experiment was freshly prepared by rubbing the quantity of histamine needed in a beaker with a glass rod into a small amount of the oil-Falba mixture until a smooth paste was formed. More of the oil mixture was added gradually with continued stirring until a sufficient amount of the vehicle had been added to make 1 cc. of the mixture contain 20 mgm. of histamine dihydrochloride. This suspension was drawn into a syringe and after vigorous shaking slowly injected through an 18 gauge needle into the subcutaneous tissue of the chest, where it was deposited as a soft lump. After the withdrawal of the needle the opening in the skin was clamped with an artery forceps so as to prevent any back leakage.

This injection was made after the dogs had been placed under nembutal anesthesia, after the femoral artery had been connected with a mercury manometer for the kymographic recording of the blood pressure, and after the blood pressure had been recorded for 10 to 15 minutes and had become stationary. Blood for hematic study was removed from the jugular vein before the injection and afterwards at more or less regular intervals in the majority of the animals used. The following components of the blood were determined: number of erythrocytes and leucocytes, volume of packed blood cells, plasma viscosity, colloid osmotic pressure, and total serum proteins. The osmometer described by Simms, Zwemer and Lowenstein (20) and the viscosimeter of Hess were used.

The shocking effects of four doses of histamine dihydrochloride were investigated, viz. 5, 10, 15, and 20 mgm. per kg. of body weight. Following the injection of the histamine suspension the blood pressure as a rule dropped gradually within 10 to 20 minutes to a level of 20 to 40 mm. Hg and stayed there for a period of several hours, showing in most instances only minor fluctuations. Where, occasionally, the blood pressure revealed a tendency to rise above 40 mm., a prompt return to the lower level was observed after gently massaging the site of the subcutaneous histamine depot. Vigorous massaging of this location five to six hours after the injection did not elicit any further vasodepressor response, indicating that the stored histamine had been exhausted.

Autopsies were performed on all dogs used in this investigation. The histological examination was restricted to a study of the thymus, lung, heart, liver, spleen, stomach, intestine, and kidney.

SERIES 1. This consisted of four dogs each of which received 5 mgm. of histamine dihydrochloride per kilo in the oil-Falba vehicle. Table 1 illustrates the responses observed as found in two dogs which are represenative of the entire group.

None of the four dogs showed any appreciable increase in the number of erythrocytes within 7 hours after the injection of the histamine. There was always an initial severe leukopenia which reverted after 5 to 24 hours into a leucocytosis, usually occurring at a time when the blood pressure was either on its way toward normal or had become normal. The viscosity of the plasma was usually slightly decreased during the first 3 to 4 hours after the injection and then showed a return to the original level. All four animals survived. The blood pressure began to rise gradually after two to four hours after the injection. The shock elicited by the subcutaneous injection of 5 mgm. of histamine dihydrochloride in an oily vehicle thus is not fatal in spite of a severe and prolonged lowering of the blood pressure.

SERIES 2. Of eight dogs injected with 10 mgm. of histamine per kilo two died within the first seven hours after the injection (6.5 hours), three dogs died during the following night, one survived for two days, and two survived for four days, when they were used in a second histamine shock experiment during which they died. The reactions observed in this group are presented in table 2 containing a summary of the data of two representative animals.

As the colloidal osmotic pressure determinations were done in only two dogs of this group the records are presented separately in table 3

TABLE 1

TIME*	BLOOD PRESSURE	ERYTHROCYTES	HEMATOCRIT	LEUCOCYTES	PLASMA VISCOSITY	DOG
minutes						
0	148	6,400,000	37	25,000	2 4	1097
30	35	5,600,000	36	4,200	2 1	
90	30	5,650,000	37	5,200	2 1	
210	35	5,950,000	38	14,500	2 25	
270	75	6,100,000	37	26,400	2 25	
330	86	6,400,000	35	27,400	2 25	
390	90	7,000,000	36	33,600	2 25	
0	118	5,600,000	35	26,300	1 75	1045
20	40	5,750,000	33	900	1 72	
80	25	4,950,000	32	1,800	1 70	
140	18	5,300,000	29	1,000	1 70	
200	30	5,100,000	32	3,900	1 65	
260	45	5 300,000	31	5,900	1 65	
320	55	5 500,000	31	5 600	1 75	
380	55	7,050,000	32	7,700	1 65	
24 hrs , 5 days		5 200,000	42	39,200	1 80	
	70	4,350,000	26	15 700	1 90	

* After administration of the histamine preparation

TABLE 2

TIME*	BLOOD PRESSURE	ERYTHROCYTES	HEMATOCRIT	LEUCOCYTES	PLASMA VISCOSITY	REMARKS
minutes						
0	125	7,800 000	51	23,600	2 45	Dog 1108 + 6 5 hrs
20	16	9,050,000	49	3,400	2 00	
70	28	8,750,000	49	4,100	1 80	
180	46	8,500,000	50	11,600	1 85	
235	24	9,100 000	54	15 000	1 85	
310	16	10,200,000	51	13,200	1 85	
0	160	8 550,000	53	13 900	1 95	Dog 1109 survived
38	40	7 850,000	49	3,100	1 65	
98	40	9 650,000	54	2 000	1 65	
186	58	8 550 000	53	6 800	1 60	
245	40	11 400,000	55	8,800	1 70	
305	32	10,500,000	56	14 500	1 80	
365	46	8,200 000	55	20,200	1 75	
425	64	10 750 000	55	20 400	1 65	
24 hrs		10 050,000	65	19,000	1 83	

* After administration of the histamine preparation

The survival rate in this series was 25%, with the majority of the fatalities occurring during the 8 to 18 hour period (40 per cent) One fourth of the animals died a relatively acute death during the first seven hours of the experiment, while 10% died a delayed death (18 to 48 hours). The hematic reactions obtained in this series are definitely more pronounced than those observed in the group of dogs injected with 5 mgm. of histamine dihydrochloride. It is noteworthy, however, that the intensity of this response does not seem to have a direct *relationship to the ultimate outcome*, as the dogs which died within the first 18 hours exhibited only a mild to moderate hemoconcentration, while this reaction reached a level of 57% in one of the two dogs which survived. The viscosity of the plasma was slightly to moderately lowered in all dogs during the observation period of 7 hours after the injection, and had returned to an almost normal level in those animals which survived 18 hours. There was a drop in the total plasma proteins during the early period, followed by a recovery above the original level in two of the dogs which died early. The colloid osmotic pressure studied in two dogs of this series exhibited a mild to moderate initial rise followed by a drop below the original level.

TABLE 3

	TIME AFTER INJECTION (MIN.)								
	0	6	75	155	205	257	321	390	433
Dog #1223, 1-20-43, 10 mgm. histamine, 7.45 kg. overnight									
Hematocrit reading (mm.)	51	47	49	49	50	51	50	51	52
Viscosity (plasma)	2.0	1.87	1.65	1.76	1.8	1.8	1.63	1.75	1.75
and temp. (°C.)	21.5	21.5	22	22	22	22	22	22	22
Colloidal osmotic press	449	471	505	507	493	469	429	371	420
Blood pressure	88	34	40	70	76	78	72	68	66
	0	19	79	136	192	251	316	383	413
Dog #1247, 1-21-43, 10 mgm. histamine, 9.5 kg. + 6.5 hrs.									
Hematocrit reading (mm.)	48	44	44	46	49	53	50	49	53
Viscosity (plasma)	2.07	1.85	1.67	1.75	1.87	1.72	1.75	1.65	1.7
and temp. (°C.)	19	19	19	19	19	21	21	21	21
Colloidal osmotic press	434	437	566	539	531	474	438	419	408
Blood pressure	128	42	54	58	68	74	50	44	24

SERIES 3. Four of the ten dogs injected with 15 mgm. of histamine dihydrochloride per kilo died during the first seven hours, five died during the following night, and one survived. Table 4 brings the main data of two dogs of this group; the first one died within 4 hours after the injection of histamine, while the second survived.

Colloid osmotic pressure determinations were made in five dogs of this series. The results obtained in four of them are presented in table 5.

The survival rate of this series was 10%. Death occurred in 40% during the early period of 7 hours, and in 50% during the second period of 8 to 18 hours Most of the dogs of this series did not exhibit any appreciable degree of hemoconcentration during the seven hour period of hematic study The highest hemoconcentration seen in this group was found in dog #1122 (table 4) The leucocytes showed the characteristic initial considerable reduction followed by a leucocytosis In one dog after five hours the leucocyte count had risen to 70,400 from an original 16,100 and a low of 6,500 The viscosity of the plasma in general showed a moderate drop during the seven hours following the injection This reduction amounted to about 25% of the original value in two dogs which died during the night

The colloidal osmotic pressure in four dogs showed a moderate to marked and progressive rise, in two instances doubling the original value In one dog, however, there occurred only a minor rise followed by a drop below the original level, which in its turn was followed by a rise, slightly above this value

TABLE 4

TIME*	BLOOD PRESSURE	ERYTHROCYTES	HEMATOCRIT	LEUCOCYTES	PLASMA VISCOSITY	SERUM PROTEIN	REMARKS
minutes							
0	140	7 450 000	45	13 200	2 25	5 97	Dog 1122 + 4 5 hrs
60	42	10 050 000	43	3 300	1 97	5 45	
130	24	7 450 000	43	1 900	2 15	5 22	
210	18	10 050 000	42	3 000	2 10	5 09	
260	0	6 750 000	41	2 800	2 25	5 81	+
0	114	6 650 000	47	13 600	1 90		Dog 1124 survived
30	24	7 450 000	41	4 200	1 65		
90	36	6 400 000	48	6 300	1 75		
150	28	7 500 000	50 5	7 400	2 00		
210	22	6 550 000	50	4 600	2 19		
270	44	7 850 000	50	14 000	1 9		
330	*30*	*7 000 000*	*50*	*13 200*	*1 85*		
420	28	8 150 000	52	17 200	1 85		
24 hrs		5 750 000	40	21 200	1 65		

* After administration of the histamine preparation

† Heart blood

SERIES 4 In this series ten dogs were injected with 20 mgm of histamine dihydrochloride per kilo None survived, eight died within the first two to six hours after the injection of the histamine, while the remaining two dogs died overnight Table 6 contains the data of two dogs, one of which died early (#1119), the other late (#1107)

The absence or mild degree of hemoconcentration in the animals of this series is remarkable Plasma viscosity and proteins showed a mild downward trend during the observation period with signs of recovery when the shock was of a more prolonged nature

PATHOLOGICAL OBSERVATIONS. For an evaluation of the organic reactions associated with histamine shock the dogs studied at autopsy were divided into three groups. In the first group were those which died during the first 7 hours of the experiment. In the second group were those which died overnight, i.e., during the 8th to 18th hour of the shock, and in the third group were those which died later. There were 14 dogs in the first group, 8 in the second, and 2 in the third. Six of the dogs included in this study had been subjected to repeated histamine shocks.

TABLE 5

	TIME AFTER INJECTION (MIN.)							
	0	30	90	155	215			
Dog #1043, 1-14-43, 15 mgm. histamine, 19 kg. + 4.5 hrs.								
Hematocrit reading (mm.)	52	64	56	58	57			
Viscosity (plasma)	1.7	1.65	1.7	1.5	1.5			
and Temp. (°C.)	18.5	18.5	18.5	18.5	19			
Colloidal osmotic pressure	446	489	683	829	892			
Blood pressure	140	72	57	46	32			
	0	26	116	181	241	311	361	411
Dog #1226, 1-15-43, 15 mgm. histamine, 10.8 kg. + overnight								
Hematocrit reading (mm.)	50	51	47	44	49	50	49	50.5
Viscosity (plasma)	1.9	1.75	1.65	1.6	1.5	1.52	1.55	1.57
and Temp. (°C.)	19	19	19	19	19	19	19	19.5
Colloidal osmotic pressure	428	478	498	526	561	583	617	647
Blood pressure	117	36	60	20	37	48	38	24
	0	12	72	132	192	252	328	374
Dog #1044, 1-18-43, 15 mgm. histamine, 8.4 kg. + 7 hrs.								
Hematocrit reading (mm.)	41	47	47	48	44	43	47	47
Viscosity (plasma)	2.27	1.95	1.95	1.7	1.65	1.55	1.55	1.7
and Temp. (°C.)	22	22	22	22	22	22	22.5	22.5
Colloidal osmotic pressure	391	409	386	436	385	224	306	430
Blood pressure	138	60	56	50	76	66	60	28
	0	24	88	168	228	293	348	403
Dog #1231, 1-18-43, 15 mgm. histamine, 15.1 kg. + overnight								
Hematocrit reading (mm.)	41	41	38	41	43	42	42	41
Viscosity (plasma)	1.95	1.9	1.97	1.87	1.8	1.8	1.75	1.85
and Temp. (°C.)	22.5	22.5	22.5	23	23	23	23	23
Colloidal osmotic pressure	347	467	444	820	619	854	593	626
Blood pressure	132	44	44	58	63	22	44	36

Group 1 The heart was dilated and flabby in the majority of cases The lungs were moderately hyperemic and showed atelectasis and cyanotic engorgement in the dependent parts The liver was moderately to markedly congested and the gall bladder wall was often markedly edematous having a greyish white area at the lower pole with engorged vessels and multiple subserous hemorrhages The spleen was contracted, greyish pink with smaller and larger tortuous, dark red, nodular projections The kidneys were hyperemic The stomach in 8 cases contained a dark brown black mucinous material There were multiple hemorrhagic erosions in the prepyloric region in one instance and large or small, round or star shaped, sharply outlined ulcers with overhanging edges and a dark bluish red colored smooth or greyish granular or ridged floor in two additional dogs These defects were located in the prepyloric parts The duodenal mucosa was swollen and displayed about 1 to 3 inches below the pylorus in many instances a dark red velvet like appearance with blood tinged liquid content The congested and hemorrhagic state of the intestinal mucosa usually decreased toward the ileum and became patchy or linear longitudinal in character occupying then a line opposite to the mesenteric attachment No appreciable congestion of the intestinal tract was found, however, in four dogs The rectal mucosa was sometimes congested and was hemorrhagic once The intestinal content was liquid and often blood tinged or blackish tarry in nature

TABLE 6

TIME*	BLOOD PRESSURE	ERYTHROCYTES	HEMATOCRIT	LEUCOCYTES	PLASMA VISCOSITY	REMARKS
minutes						
0	135	9,100,000	53	10,800	2 3	Dog 1119 + 5 hrs
30	50	7,200,000	46	2,600	1 97	
90	30	8,650,000	55 5	9,800	2 25	
150	30	8,000,000	50 5	10,000	1 97	
210	30	8,600,000	52	14,400	2 0	
270	20	8,600,000	50 5	19,000	1 98	
300	10	9,200,000	52	16,400	2 0	
0	140	7,150,000	46	20,700	2 7	Dog 1107 + overnight
45	24	7,100,000	47	3,100	2 47	
105	40	8,100,000	50	3,100	2 25	
190	34	8,500,000	53	13,000	2 25	
250	34	8,750,000	51	17,400	2 27	
310	38	6,800,000	52	15,600	2 2	
370	40	8,550,000	51	23,200	2 15	
430	50	8,600,000	50	23,600	2 9	

* After administration of the histamine preparation

Group 2 The thymus was dark red and hemorrhagic in three dogs The heart was always firmly contracted and the myocardium was pale red The lung was congested and was partially or completely collapsed in four cases in which considerable amounts of a dark brown turbid fluid filled the pleural cavities The abdominal cavity of one animal contained large amounts of a dark brown turbid fluid and in two cases a blood tinged watery material The liver was either normal in appearance or slightly congested and the gall bladder wall was edematous or normal The spleen was contracted and in several cases *contained dark red nodules of hemostasis embedded in a greyish pink, dry pulp* The kidneys were normal The stomachs of two dogs showed ulcers in the prepyloric region of the lesser curvature *Hemorrhages* in the mucosa of the pyloric ring were found once The duodenal mucosa showed a hemorrhagic imbibition starting about one inch below the pylorus in one instance. In several other cases the mucosa was congested and in about half the dogs the mucosa was grossly normal. The small intestine was either normal or showed a slightly hyperemic mucosa only once containing numerous hemorrhages. The colon and rectum more often revealed a congested mucosa complicated in three instances by hemorrhages and a bloody liquid content. The subcutaneous tissue had a cyanotic appearance.

Group 3. The macroscopic findings were in general similar to those of group 2.

Microscopical Data: Group 1. Subepicardial or subendocardial hemorrhages were present in two hearts, combined in one instance with a subendocardial lymphocytic and monocytic infiltration, while associated in the other case with swollen myocardial arterioles. These arteriolar lesions were present in three additional hearts. The lungs were moderately congested; a few had focal hemorrhages, atelectases and emphysema. The liver exhibited a considerable or even highly excessive edema and congestion, especially in the pericentral, and sometimes also in the periportal tissue. The liver cells in the pericentral zone were atrophic or vesicular or foamy and were sometimes surrounded by leucocytes. The gall bladder exhibited a large blister-like edema of the subserosa not infrequently associated with hemorrhages and leucocytic infiltrations. In four cases there were focal and sometimes extensive necroses of the entire wall. The spleen showed a pulp containing erythrocytes in small number except in those restricted areas in which the sinuses and pulp were packed with erythrocytes and where a hemangioma-like structure prevailed. This condition was associated in one instance with necrotic changes and in a second instance with a small rupture of the capsule. The kidney was hyperemic and showed a vacuolation of the tubular epithelium. The gastric mucosa was edematous in several instances, in others defects were present filled with erythrocytes and involving only the mucosa, while the submucosa revealed once thrombosed vessels beneath the ulcers. Occasionally submucosal hemorrhages were present. The duodenal mucosa showed a more or less marked engorgement of the mucosal vessels, sometimes associated with hemorrhages into the tips of the folds. Similar but usually less extensive vascular conditions were found in the small intestine and rectum.

Group 2. The heart in three instances showed swollen myocardial arterioles with thickened walls bulging into the contracted or collapsed vascular lumen. Perivascular lymphocytic infiltrations existed in one dog. The lungs were congested, edematous and contained hemorrhages. The stomach revealed in three cases small or large ulcerations. In one instance the ulcerative defect extended into the muscularis which was superficially necrotic and formed the floor of the ulcer. The submucosa was highly edematous and congested and contained extensive hemorrhages within necrotic tissue. These lesions extended far beneath the periphery of the ulcer. The overhanging mucosal edges were hyperemic and contained hemorrhages. The vascular walls of the submucosal vessels were swollen and their lumina were filled with hyaline or leucocytic thrombi, while large mononuclear cells lined the lumina. The duodenum showed necroses in the superficial parts of the mucosa as well as edema and congestion of the mucosa. Similar changes occurred in the small intestine to a minor degree, whereas they were sometimes much more extensive in the rectal mucosa. The kidney was congested and contained interstitial hemorrhages with vacuolar degeneration of the tubular epithelium. The splenic pulp in general contained a small amount of erythrocytes, only in more or less circumscribed areas there existed a massive accumulation of erythrocytes with distension of the sinuses producing the picture of a hemorrhagic infarction.

Group 3. The histological findings were similar to those seen in group 2. There was a large epicardial hemorrhage in the heart of one of the dogs, which was associated with a swelling of the arteriolar walls and a large myocardial necrosis. The edematous lung of the second dog exhibited a thrombosis of a branch of the pulmonary artery with anemic infarction of the lung tissue.

COMMENT. The recorded data show that it is possible under the experimental conditions stated to produce a standardized and graded histamine shock in dogs, as demonstrated in table 7, which shows the variations found in the different series as to mortality rate and duration of survival.

This information is essential for the testing of therapeutic agents, as their efficacy depends in part upon the severity of the shock which in turn is related to the shock dose and the length of time after its introduction. An application of these studies for the indicated purposes has shown that therapeutic agents effective against standardized and graded histamine shock increase both the survival period as well as the survival rate of the shocked animals (10).

It is remarkable that the majority of the dogs subjected to histamine shock did not show any appreciable degree of hemoconcentration at any time. Some of the dogs exhibited this condition for some time during the course of the shock, but rarely in a progressive form, while in a few there was even a reduction in the number of erythrocytes, suggesting the development of a hemodilution. This last type of reaction was noticed particularly at the twenty-four hour period irrespective of the preceding type of response. Similar observations have been made previously in dogs subjected to histamine shock and cold shock produced by the application of dry ice to the back (8) (9). These animals exhibited directly after the production of the shock a progressive erythropenia and leukopenia with fatal outcome within 8 to 48 hours Bosse, Gross, and Hagan (1) recently reported similar evidence obtained in rabbits subjected to burn shock elicited by scalding. Hemoconcentration caused by increased capillary permeability and regarded by many investigators as the basic mechanism in the dynamics of shock (15) is apparently not a hematic reaction consistently associated with this condition (24). The vascular and hematic reactions in shock are evidently of a much more complex nature.

TABLE 7

SERIES	DEATH 0–7 HRS	8–18 HRS	19–48 HRS	SURVIVAL
	per cent	*per cent*	*per cent*	*per cent*
1				100
2	25	40	10	25
3	40	50		10
4	80	20		

Similar difficulties are encountered when an attempt is made to correlate the hematocrit values with those obtained for plasma viscosity, serum colloid osmotic pressure, and total serum proteins. If it is correct that shock is characterized by a loss of serum proteins into the tissues with a resulting reduction in colloid osmotic pressure, definite relations should exist between the factors mentioned. While there was a certain general tendency of the plasma viscosity to decrease with the duration of the shock, this was by no means a consistent observation, nor was it associated with a corresponding reduction in the amount of total plasma proteins. The plasma viscosity is not only dependent upon the amount and ratio of the various plasma proteins (7), but also upon their state of dispersion and hydration (12)

It is especially noteworthy that the colloid osmotic pressure of the serum, determined with undiluted serum against water, exhibited in most instances a considerable and often progressive increase up to the time of death, irrespective of the movement of the hematocrit values and of the plasma viscosity. The colloid osmotic pressure of the serum proteins depends on the same factors which influence the viscosity, in addition to the effect which substances adsorbed on the surface of the particles may exert in this direction (17). Mention must be made of the fact that the colloid osmotic pressure of serum changes upon standing and is definitely modified by a dilution of the serum with saline (14). The last two factors most probably play a rôle in all those determinations of the colloid osmotic pressure of serum in which the collodion bag procedure is used. It is therefore uncertain whether our values of colloid osmotic pressure are directly comparable to those of other investigators.

More recent observations indicate, moreover, that the colloidal osmotic equilibrium of the blood sometimes reacts to the introduction of exogenous or endogenous colloidal solutions in an apparently paradoxical fashion. Goudsmit, Binger and Keith (5) thus reported that the injection of acacia solution into nephrotic patients causes in an appreciable percentage a drop in colloid osmotic pressure. Similarly, Harkins, Boals, and Brush (6) noted that the intravenous introduction of human or canine serum resulted in a hemoconcentration. The control of the extra-cellular water metabolism by the plasma proteins and exogenous colloids through their osmotic and water binding powers is evidently not yet sufficiently understood to form a basis on which an intelligent colloid therapy of shock can be developed.

The pathological observations made suggest, moreover, that at least during the relatively early period of histamine shock (death within the first seven hours), increased capillary permeability and colloidal osmotic disturbances of the plasma cannot represent the main cause of death, as manifestations indicative of these conditions in an appreciable percentage of such animals were only mildly developed. The constant occurrence of a dilated heart as well as the presence of myocardial hemorrhages and myocardial arteriolar swelling with collapse or contraction of the walls point toward a cardiac mechanism of death. Such discrepancies apparently led Klisiecki and Holobut (11) to conclude that histamine injures the wall of the left ventricle directly, thereby causing a drop in blood pressure. Meessen (13), who observed similar myocardial lesions after histamine shock, attributed the failure of the heart to a primary coronary insufficiency.

It is obvious, on the other hand, that circulatory collapse subsequent to an immobilization of large amounts of blood in the liver, intestinal mucosa and kidney, together with direct loss of hematic fluid into the intestinal lumen and into the serous cavities, provided the fatal mechanism in some of the dogs which died during a later period. It may be mentioned in this connection that the spleen took an active though not prominent part in the hemostasis, as evidenced by the occurrence of sometimes extensive dark red nodulation caused by an infarct-like accumulation of blood in the pulp and sinuses of some parts of the spleen. The liver of the dog, with its venous barrier, and the canine spleen, with its storage capacity for about one fifth of the total blood volume, probably play a greater rôle in the shifts of blood during shock than that which they can assume in man, whose spleen can retain only one twentieth or one-thirtieth of the total blood volume (21)

Hemorrhage from eroded vessels in the gastric ulcers observed in some of the dogs may have contributed to the fatal outcome in a few However, it is unlikely that this complication was of any great importance in this respect The occurrence of gastric or duodenal ulcers following the injection of histamine has been reported previously (4) (19) (22) The increased production of gastric secretion (2) (23) as well as the circulatory changes in the gastric mucosa and submucosa are apparently responsible for these manifestations which possess some importance in regard to the genesis of peptic ulcer in general

CONCLUSIONS

1 By the injection of increasing doses of histamine dihydrochloride suspended in a cottonseed oil-Falba base it is possible to produce a standardized and graded shock in dogs

2 Considerable hemoconcentration was not a characteristic hematic manifestation in many of the dogs

3 The plasma viscosity decreases only mildly to moderately during shock, while the colloid osmotic pressure of the serum increases considerably

4 Early deaths during histamine shock are apparently as a rule cardiac deaths, while animals with delayed deaths exhibit manifestations associated with increased capillary permeability

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THE INEFFECTIVENESS OF ADRENAL CORTEX EXTRACTS IN STANDARDIZED HEMORRHAGIC SHOCK[1]

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In order to evaluate the efficacy of remedial measures in hemorrhagic shock it is essential to have a standard procedure by which it can be produced with predictable frequency. To be practical, the method must be simple as well as effective.

Previous attempts to standardize hemorrhagic shock have followed two general trends: (*1*) Definite volumes of blood per kilo, which are fairly large but immediately consistent with life, are removed at more or less definite rates of bleeding and the subsequent effects on blood pressure, blood volume, revival or survival of the animal, etc , are compared in untreated or treated animals. (*2*) Animals are bled variable volumes required to reduce arterial pressures to some arbitrary "shock" level, usually 50 or 60 mm. Hg, and the effects of remedial agents or blood substitutes are studied by following the changes in arterial pressure, cardiac output, blood volume, hemoconcentration, blood chemistry, time of survival, etc.

Our experimental work during the past 2½ years has confirmed the conclusion of others that the first procedure is unsatisfactory because untreated dogs show great variability in their reactions to and tolerance of definite blood losses computed on the basis of body weight. This is not surprising In addition to the operation of variable compensatory mechanisms, recent studies of Bonycastle and Cleghorn (1) have reconfirmed the fact that blood volumes in stray dogs vary considerably, in their series from 66 to 107.5 cc./k. with a mean of 82.1 cc /k. If the procedure is used, the volume of blood withdrawn should be based on predetermined circulating volumes, but even this ignores the variable quantities stored in blood depots and the individual variations in compensatory mechanisms.

Our early experimental studies (2) had indicated that the development of irreversible circulatory failure—generally associated with congestive and hemorrhagic changes in the duodenum and endocardium—depends both on the degree and duration of post-hemorrhagic hypotension. The possibility was therefor investigated whether a practical method for producing standardized hemorrhagic shock might be evolved through creation of successive periods of moderate and severe hypotension by regulated bleeding It was postulated that the more prolonged and safer period of moderate hypotension might take care of the time element while the more effective period of severe hypotension could be kept short enough to prevent untimely cardiac or respiratory failure.

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In a preliminary survey of many experiments in which these intervals varied considerably, Werle and Wiggers (3) found that an initial period of moderate hypotension (*ca.* 50 mm. Hg) lasting 90 minutes followed by a 45 minute period of severe hypotention (*ca.* 30 mm. Hg) always resulted in irreversible circulatory failure. These conclusions were based, however, on results from dogs under various types of intravenous anesthesia and submitted to variable operative procedures necessary for different types of dynamic studies. In some the animal's blood had been rendered noncoagulable by injections of heparin, in others carotid arteries had been occluded or one or both vagus nerves had been cut, etc. We have therefore systematically explored use of the method on animals which were all anesthetized with morphine and minimal effective doses of sodium barbital (175–200 mgm./kilo) and subjected to essentially the same minimal operative procedures. All retained potent sinus and aortic reflexes. Efforts were made to maintain the initial rectal temperature throughout the experiment by regulation of external heat. Bleeding was started about one hour after induction of anesthesia.

We found that shorter intervals than those suggested did not produce irreversible shock in a sufficient proportion of animals to be serviceable and we therefore retained the criteria suggested by Werle and Wiggers. We modified the technique as follows: The mean pressure of the dog is lowered to 50 mm. Hg in one stage by withdrawal of as much blood as necessary at a rate of 50 cc./min., reducing the rate a trifle as lower pressures are approached. The blood is drawn into heparin solution[2] and kept cool until required for reinjection. Usually a little more blood must be drawn periodically in order to maintain such a 50 mm. level. At the end of 90 minutes the mean pressure is cautiously reduced to 30 mm. Hg by slow bleeding (4–5 cc./min.). Whenever pressure rises or falls ±5 mm. from this level, a little blood is either withdrawn or reinfused. An ordinate writer on the smoked drum by which mean pressures may be constantly gauged is almost a necessity.

Our experience indicates that with the bleeding rates stated, blood pressures are usually reduced to 50 mm. by bleeding approximately 40 cc./k. and subsequently to 30 mm. by an additional bleeding of 3 to 5 cc./k. However, these are only useful as rough guides since considerable variation exists. The net total bleeding in all our dogs ranged from 20 to 56 cc./k., with an average of 37.2 cc./k.

Our experience with the method in an additional 50 dogs enables us to point out a number of difficulties and certain expedients by which they can be overcome. During or after the initial faster hemorrhage, Traube-Hering waves[3] frequently develop. Since the magnitude of these variations may equal 20–30 mm., the question arises as to how an average 50 mm. mean pressure should be read. Since, after their disappearance, mean pressure generally stabilized at a level midway between the crests and troughs of these larger waves and since planimeter measurements gave a similar average, this midpoint represents the mean fairly well During the 50 mm period the pressure often tends to rise somewhat Whenever this equals 8 mm and stabilizes there for about 1 minute, additional blood is withdrawn in order to reduce pressure to the 50 mm standard Pressure rarely falls except toward the end of this period If this occurs within 5 minutes of the time set for the second bleeding no attempt is made to restore pressures by reinfusion of heparinized blood, but the period of drastic hypotension is reduced commensurate with the degree and duration of the previous decline

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[2] We are greatly indebted to Roche-Organon Co., Rahway, N. J., for a generous supply of Liquaemin used in these experiments.

[3] If, as seems probable, these variations are due to rhythmic variations of vasomotor tone, their presence after bleeding indicated that the vasomotor center is acting periodically during the period of post-hemorrhagic hypotension.

The second bleeding by which pressure is reduced to 30 mm must be slow, and trouble is avoided if pressures are not allowed to decline below this level With this precaution, animals withstand such hypotension well for 15–30 minutes Respiratory rate and depth generally remain increased or at least are not subnormal During the last 10 or 15 minutes, an animal must be constantly watched The danger signals are slight progressive decline of arterial pressure, manifest slowing of the heart and diminishing rate and/or depth of respirations These may occur separately or together, and once developed may quickly result in death Figures 1 A and 1 D are examples of rapidly declining heart rates Therefore, as soon as any of these signs appear, mild artificial respiration and reinfusion of some blood must be instituted at once Small infusions (20–30 cc) often suffice, but occasionally 100 to 200 cc are required Lately we have adopted the practice that when an infusion of 100 cc of blood does not promptly increase heart rate and blood pressure, the aortic pressure and coronary flow are augmented directly The most effective procedure consists in injecting 20–75 cc of Ringer's solution into the aorta *via* a carotid artery under a pressure of about 200 mm Hg This temporary increase of aortic pressure probably supplies a flow of mixed blood and saline through the coronary arteries sufficient to increase the pumping action of the ventricles, and this immediately mobilizes blood from the right heart into the pulmonary and systemic circulations, thus restoring a circulatory balance The addition of adrenalin or similar agents which we previously found useful (2) is unnecessary and only complicates the interpretations following revival With these precautions, animals rarely die except through lack of watchful attendance during this phase of the experiment

At the termination of the 30 minutes period, blood previously drawn from the animal is warmed, adequately filtered and reinjected at a rate which should not exceed 50 cm /min at the start and should proceed more slowly as arterial pressure rises to higher levels The infusion rate should be reduced as soon as arterial pressure no longer continues to rise and infusions should be stopped at once if it begins to fall, for this is associated with rapid elevation of venous pressure and signifies over distention of the right heart

Methods After development of our standard technique, we followed the course of hemodynamic events and the incidence of survival in a new series of animals For this purpose, central arterial pressure was recorded and right atrial and intrathoracic pressures were read on a damped water manometer during expiratory phases In this way, effective atrial pressures were calculated by algebraic difference

However continuous registration of mean femoral pressure by a damped Hg manometer is sufficient for utilization of the method By placing a little heparin solution in the cannula it is not even necessary to use anticoagulant solutions with careful operative technique, normal saline is just as good as other salts commonly employed as anticoagulants In this event, it is preferable to determine heart rates by simultaneous registration of an electrocardiogram or by auscultation since Hg manometer oscillations are not always reliable

Results Data from a control series of 21 dogs are given in table 1 The total net volume of blood withdrawn ranged from 24 to 53 5 cc /kilo and averaged 44 4 cc In this group, four of the earlier animals died of cardio respiratory failure during the 30 mm hypotension period and necropsies revealed intense congestive and hemorrhagic changes of the gut and presence of subendocardial hemorrhage They would probably have reacted unfavorably to rein fusion. The other 17 animals immediately reacted well to reinfusion of the withdrawn blood (heparinized). As a rule, mean arterial pressure did not return quite to normal, but central pressure pulses had a normal contour. In 5 experiments complete restoration of mean pressure occurred and in 2 experiments mean pressure even exceeded control levels. Variations occurred as regards the subsequent course. In 7 animals mean arterial pressure started on a rapid downward course ½ to 1½ hours after reinfusion and the central pressure pulses showed characteristic changes of circulatory collapse. This *precipitant type* is illustrated by the graph of figure 1-A. A second group of six dogs maintained

TABLE 1

Results of control group showing amount of anesthetic agent, rectal temperature, net hemorrhage, and intestinal changes

EXP. KA	BARBITAL	RECTAL TEMP.		NET TOTAL HEMORRHAGE PER KILO	INTESTINAL CHANGES
		Start	End		
Non-shock					
	mgm./Kilo				
113	175	38	37.4	39.4	+
139	200	37.6	37	51.7	?
159	175	35	37.8	52.8	0
161	110	38	39	45.1	+
Precipitant shock					
106	175	37.3	37	37.0	++
128	175	38.7	37	24.0	++
132	175	35.9	32.5	39.5	++
141	170	35.9	30.5	43.0	?
164	180	35	37.5	37.5	++
165	175	35.5	40	40.0	+++
166	175	35	40	53.5	+++
Delayed shock					
109	175	38	39	34.6	+
125	175	34	34.5	33.9	0
140	165	35.9	33.5	45.6	+
167	180	36	37	41.0	+
168	183	37	36.9	46.7	++
169	161	36	37	41.1	?
Accidental deaths toward end 30 mm. hypotension					
105	175	38.5	39	34.6	++
107	175	37.5	38	44.4	++
108	175	38.0	40	31.3	++
110	175	41	41+	33.1	++

Total experiments, excluding 4 accidental deaths, 17, percentage showing circulatory failure, 76.5.

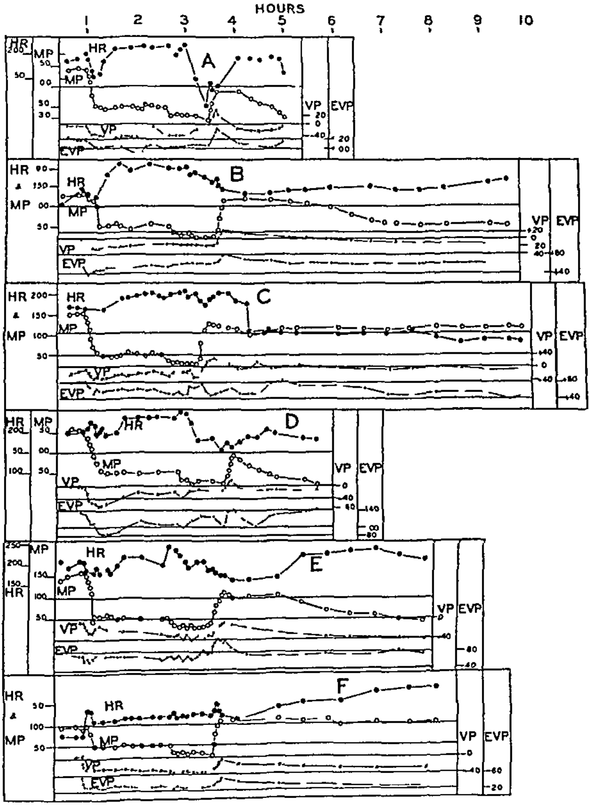

Fig 1 Plots of heart rate (H R), mean arterial pressure (M P) actual auricular pressure (V.P) and effective venous pressure (E V P) during moderate post hemorrhagic hypotension (50 mm) drastic hypotension (30 mm) blood reinfusion and precipitant (A D) delayed (B E) and non-shock (C F) reactions which follow A B C, *controls*, D, E F, *dogs treated with ACE*

mean arterial pressures of at least 90 mm. Hg for 2 to 2½ hours after which pressure more gradually declined to shock levels for the succeeding 2 to 4 hours. This *delayed type* is illustrated by the graph of figure 1-B. In both types, pronounced congestive and hemorrhagic changes in the duodenal mucosa and spotty subendocardial hemorrhages were usually found at autopsy. In a third group of 4 animals, arterial pressures were maintained at normal levels for 4 hours or *more, while the pressure pulses retained* good form and normal details. This type is illustrated by the graph in figure 1-C. Since our general experience indicated that animals which retain essentially normal arterial pressures for 4 hours do so for 8 to 10 hours, our experiments were generally terminated at this time. Since, furthermore, very slight or no pathological changes were seen at autopsy, we counted these as *non-shock* animals despite the fact that such operated and continually anesthetized dogs died subsequently. They were non-shock animals in the sense that dynamic equilibration was maintained after restoring the blood lost by bleeding.

A summary of the control animals in this series reveals that 13 out of 17 dogs or 76.5% died of hemorrhagic shock. The percentage is increased if we include the 4 animals which, to judge from postmortem changes, would probably have responded unfavorably.

THE EFFECTS OF ADRENAL CORTICAL EXTRACTS. We attempted to evaluate the efficacy of adrenal cortex preparations by determining whether animals treated or pretreated with such extracts show a greater tolerance for successive periods of moderate and severe hemorrhagic hypotension to which 76.5% of our untreated animals succumbed despite reinfusion of all the withdrawn blood. Comparisons of the percentage of recoveries, the course after reinfusion of blood and the changes observed at necropsy all serve as criteria.

Since it remains questionable which of the substances isolated from the adrenal cortex might prove potent our studies were restricted to use of potent extracts of the whole cortex (ACE). Three such preparations were used, viz. (*a*) an experimental preparation furnished by the Research Laboratories of Parke-Davis and Co., and containing 40 grams of fresh cortex per cc., (*b*) a preparation (735–736) kindly supplied by Dr. Kendall of the Mayo Foundation, each cc. equivalent to 150 mg. adrenal cortex and (*c*) a solution furnished by Upjohn Co., standardized to contain 2.5 rat units per cc.[4]

Twenty-five dogs were bled as before and similar periods of post-hemorrhagic hypotension were maintained. Of these, 9 had received an intramuscular injection of 10 cc. extract on the previous afternoon. Immediately after the first large hemorrhage, all received a continuous slow drip infusion of diluted extract which extended through most of the 50 mm. hypotension period. The beginning of such infusions was delayed until this time in order to prevent the loss of injected extract in the withdrawn blood. However, in two experiments (111, 114) the extract was administered in larger divided doses and in three animals

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[4] We are indebted to Dr. Kamm of Parke-Davis and Co., Dr. Kendall of the Mayo Foundation and Dr. Wenner of the Upjohn Co. for furnishing generous supplies of ACE for these experiments.

(127, 131, 133) 5 cc of extract was given with the intravenous anesthetic instead of during the previous afternoon

TABLE 2

Results of cortin groups showing amount of anesthetic agent, rectal temperature, net hemorrhage, intestinal changes, dosage and preparation of cortin

EXP KA	BARBITAL	RECTAL TEMP		NET TOTAL HEMORRHAGE PER KILO	INTESTINAL CHANGES	ADRENAL PREP	PRIMING DOSE	TOTAL DOSE
		Start	End					
				Non shock				
	mgm/kilo						*cc*	*cc*
116	210	36 9	37 5	45	0	Parke Davis		20
119	175			40	?	Kendall		17
135	175	38 1	39 8	41 3	+	Kendall	10	20
137	175	37	36	31	?	Kendall	10	20
145	170	37	32	36 4	+++	Kendall		10
				Precipitant shock				
115	170	37	36 8	49	+++	Parke Davis		18
117	180	38	36	38 3	++	Upjohn		20
118	175	36	38	41 7	+	Upjohn		17
121	175	35 8	38 8	33 3	+	Upjohn		20
123	165			21 4	+	Kendall		22
124	175	37 2	36	36 6	++	Upjohn		20
126	175	36 9	36 6	33	++	Parke Davis		15
131	160	38	36 5	24 5	+++	Parke Davis		20
133	170	40	38	32 3	0 or ?	Kendall	10	35
136	225	37	38 2	41 8	++	Upjohn		10
143	175	36 8	35	49 3	+++	Kendall	10	20
144	180	36 1	34 2	43 7	+	Kendall	10	20
146	175	37	32	20	+++	Upjohn	10	20
				Delayed shock				
111	200	37 5	38 5	40	++	Parke Davis	10	31
122	173			54	+	Parke Davis		18
				Accidental deaths at 30 min hypotension				
112	125	38	?	37 0	+	Parke Davis	20	25
114	165	39 8	40	41 0	+	Parke Davis	10	25
127	175	37	36 5	50	++	Kendall		20
129	200	35 5	36	50	++	Upjohn		20
142	180	37	36 5	40	+	Kendall		10

Total experiments, excluding 5 accidental deaths, 20, percentage developing circulatory failure, 75

Results A survey of the streamlined data incorporated in table 2 shows that, again excluding 5 accidental deaths during the 30 min hypotension period, 15 of 20 dogs or 75% died from hemorrhagic shock after reinfusion of withdrawn blood. This is almost the same incidence found in untreated dogs.

Plots of the course of events in separate experiments showed no discoverable differences. Of the 15 dogs which developed shock after reinfusion of blood, 13 were of a precipitant type, illustrated by figure 1-D, 2 displayed a delayed course illustrated by figure 1-E, and 4 recovered as illustrated by figure 1-F. If any difference in the course of events were claimed, it would have to be that a precipitant type of shock occurred far more frequently in these treated animals. All of the animals that died showed intensive changes in the duodenal mucosa and endocardium. Since quantitative evaluation is hazardous, it may be merely an impression or an accident that the congestion, edema and tendency to duodenal hemorrhage seemed to be even more intense in treated animals. If, as has been claimed, adrenal cortex extracts act favorably on capillaries, the reverse might have been expected.

Effects on venous pressures. If, as has been claimed, the beneficial action of adrenal cortex extracts (ACE) consists in maintenance or restoration of capillary tone or permeability, it may be anticipated that venous return and venous pressures would be benefited. In a previous report (2) the conclusion was reached that a lowering or progressive decline of effective venous pressure (EVP) accompanied the progressive decline of arterial pressure in only 4 out of 12 experiments. The present series of animals treated in a more standardized manner offers additional information as to the course of venous pressures in untreated animals and by comparison possible differences in animals treated with ACE. Comparison of venous pressures at the beginning of experiments show that 40% of primed animals had an EVP over 80 mm. saline against 68% of unprimed animals. Obviously, pretreatment does not increase venous pressures. The following details may be recorded in substantiation of this conclusion. In 10 dogs pretreated with ACE, EVP ranged from 40–59 mm. in 2; 60–79 mm. in 4; 80–99 mm. in 2; 100–130 mm. in 2; whereas in 24 unprimed dogs EVP ranged from 40–59 mm. in 2; 60–79 mm. in 6; 80–99 mm. in 6; 100–130 mm. in 8 and from 138–180 mm. in 4.

With the first large hemorrhage, which reduced mean arterial pressure to 50 mm., actual venous pressures (VP) generally fell, as shown in various plots of figure 1. However, as previously pointed out, intrathoracic pressure likewise changed as a result of the decreased thoracic blood content and changes in respiratory activity. In this series, intrathoracic pressure (during expiration) was not affected in 7, increased in 9 and decreased in 18 experiments. As shown by the plots of figure 1, this affected the magnitude but not the trend of changes in EVP.

At the end of the 50 mm. period, VP remained lower than control values in 6 untreated dogs; it was the same in 6 and a trifle higher in 2. In the same group of 14 dogs, EVP was lower in 9, the same in 2 and higher in 3 cases. Of 21 dogs which had received ACE up to the end of this 50 mm. period, VP remained below control values in 14, was the same in 6 and a trifle higher in 2 cases. EVP was lower in 16, the same in 2 and higher in 3 cases.

Such results show (*1*) that on the whole EVP are definitely reduced during the whole period of moderate hypotension despite some tendency to rise as illustrated in graphs 1-A, B and D, and (*2*) there is no evidence that ACE favors such recovery (cf. fig. 1, D-E-F).

In comparison with the end of the 50 mm. period of hypotension, venous pressures showed variable trends during the subsequent 30 mm. period. This is partly due to the fact that pronounced slowing of the heart rather than rate of venous return flow may dominate the venous pressures. Such elevations of VP and EVP are illustrated in figure 1-A and D. In order to gain any information from venous pressures as regards rate of return flow, comparisons must be made before significant cardiac slowing sets in. Even when this was done could any consistent directional trend or any determining effect of ACE injections be found in untreated dogs. Thus, in 13 untreated dogs VP decreased in 4, remained unaltered in 6 and rose in 3 cases, while EVP decreased in 6, remained the same in 2 and rose in 5 cases. Similarly, in 13 dogs treated with ACE, VP decreased in 7, was the same in 3 and rose in 3 cases, while EVP decreased in 4, was unchanged in 3 and rose in 6 cases.

Immediately after reinfusion of heparinized blood, EVP increased over controls except in 3 animals in which it returned just to normal and in 5 in which it remained slightly lower. The latter was due to predominant changes in intrathoracic pressure. The subsequent course is best considered in relation to development of non-shock, precipitant shock and delayed shock in untreated and treated animals. In one untreated non-shock dog shown in the plot of figure 1-C, VP remained a little above normal to the end; in the other it was 20 mm. lower than normal. In both EVP was lower. In 4 ACE treated dogs VP gradually decreased but remained above normal. EVP, as illustrated in figure 1-F, also decreased in two, but was above normal in the other two. The most interesting fact in this group of experiments was the maintenance in two of normal circulating balance for hours despite progressively decreasing VP and EVP.

Of the animals which developed a precipitant type of shock, venous pressures could be accurately followed to the end in 4 untreated and 11 ACE treated dogs. Examples are shown in figure 1-A and D. In the untreated dogs VP was below control value in 2 and above this in 2 cases; while EVP was at or above control values in 3, and below in one (plotted as fig. 1-A). In the ACE treated dogs, VP was below normal in all except 3 cases, and EVP was less than normal in all except 5 cases. One of the latter is plotted as figure 1-D. In short *1*, the development of precipitant shock after reinfusion of blood is not related to the trend of VP or EVP and *2*, the trend is not affected by ACE therapy.

Conclusions. On the basis of the incidence of irreversible circulatory failure, the course of its development, changes in venous pressures and pathological appearances at necropsy, we are forced to conclude that pretreatment or treatment of dogs with large doses of ACE has no discoverable favorable effect in hemorrhagic shock.

DISCUSSION. The results of this investigation accord with our recent observations on the ineffectiveness of ACE in fulminant shock (3, a). They are also in agreement with the conclusions of many investigators. Since they are at variance with those of others, an attempt ought to be made to discover the cause of these differences. It is particularly important to evaluate the extent to which different procedures and criteria have affected the conclusions reached.

The use of adrenal cortex extracts was, we believe, first suggested by the following series of discoveries: *1.* Many physiological manifestations of shock and some pathological changes discovered at necropsy resemble those which occur during terminal stages of double adrenalectomy. *2.* In such animals adrenal cortex preparations have a favorable effect in restoration of water, electrolyte and carbohydrate balances, on the recovery of arterial pressures and in prevention of death. *3.* Minor insults, without effect on normal animals, induce shock in adrenalectomized dogs and this can be ameliorated, prevented or cured by adrenal cortex preparations.

However, despite many similarities between the terminal manifestations of shock and of adrenal cortex insufficiency it remains to be demonstrated that adrenal insufficiency occurs in clinical or experimental types of shock in non-adrenalectomized dogs or men. The observations that the adrenal cortex is congested, hemorrhagic or depleted of its lipoids (4, 5) or that the distribution or excretion of adrenal steroids is changed (6) do not constitute sufficient evidence for such an hypothesis. In contrast to the dramatic revival of dogs moribund after adrenalectomy, no one has succeeded in halting the irreversible course of hemorrhagic, traumatic, surgical or toxemic shock by administration of cortex preparations *in nonadrenalectomized animals.* Maintenance of an adrenalectomized dog does not mean that the animal is entirely normal. Recent observations of Swingle *et al.* (7) clearly showed that the effectiveness of adrenal steroids in ameliorating shock depends on the steroid or steroids previously used to maintain the animals. But even an adrenalectomized animal, maintained on ACE has less hormonal reserve than an intact animal and could not have the reputed capacity of increasing its output of adrenal cortex hormones. Admitting that this could be rectified by administration of ACE in adrenalectomized animals and that this increases resistance to shock-producing agents, the corollary does not follow *ipso facto* that such supplementary hormones are necessary or useful in non-adrenalectomized animals. Therefore, the employment of adrenal cortex preparations cannot be regarded as replacement therapy.

A second basis for their employment rests on two hypotheses: *1,* that damage to capillaries constitutes an important factor in determining the irreversible state of shock and *2,* that adrenal cortical hormones favorably affect capillary permeability and tone. In support of the latter, experiments of Swingle and his associates have long been quoted despite the fact that they have emphasized repeatedly (8) that "the cortical hormone *per se* is probably not directly concerned with capillary permeability." In 1938, however, they (9) produced all the circulatory changes of shock in *adrenalectomized* dogs by intestinal stripping without incurrence of characteristic electrolyte changes. They explained this by failure of capillary tonus, but produced no evidence for this explanation. More recently, Swingle *et al.* (10) reported that serum infusions lead to circulatory collapse and edema in adrenalectomized dogs, but that this is prevented by use of ACE or DCA. They then reversed their previous interpretations and concluded that adrenal cortex preparations exert a favorable effect on capillary permeability. It should be noted in passing that the edema of the eyes, lips, ears, legs, abdominal walls and scrotum which they produced have hitherto not been described as manifestations of shock in intact animals. Cope *et al.* (11) recently reported that the protein content and enzymatic activity of cervical lymph obtained by the McCarrell method increases after adrenalectomy. This they interpret as evidence of increased capillary permeability in the regions drained. It would be interesting from the viewpoint of the permeability problem to determine whether similar changes also occur in experimental shock. However, since such loss of protein to the lymph would normally be returned to the blood stream, it could not be a factor in blood protein depletion unless considerable edema occurred.

In a recent review (12), attention was called to the paucity of experiments planned to test the effect of adrenal cortex preparations on permeability, directly. Since that time more such experiments have been reported In addition to earlier studies of Menken (13) and of Freed and Lindner (14), the Michael Reese investigators (15) reported that corticosterone which prevents the increased permeability of capillaries produced by leucotaxin does not prevent the increase due to peptone, whereas ACE does so. However, they also report (14, 16) that DCA which does not prevent edema of a venous-occluded leg nor the increased permeability caused by leucotaxin is more potent than ACE in preventing shock due to such venous obstruction. Apparently, this type of shock is benefited by adrenal factors otherwise than through changes in capillary permeability. Root and Mann (17) recently described the behavior of hepatic and mesenteric capillaries studied by the Knisely technique They observed the appearance of newly functioning capillaries, and dilatation, congestion and stasis both after intestinal manipulation and following release of a constriction about the limb. However, they noted no beneficial effects from ACE (Kendall preparations). Our own gross necropsy findings and microscopic examinations of fresh preparations immediately after death showed that if the congestion and hemorrhage observed are due to alterations of capillary permeability or tone, such changes are not generalized during hemorrhagic shock, but are limited to the duodenal mucosa and endocardium.

A conservative attitude would be that, while it is possible that capillary damage contributes to the irreversibility in shock and that adrenal cortex factors can favorably counteract the damage which occurs in non-adrenalectomized animals, neither premise has been proved. Lacking concordant evidence that adrenal cortex preparations can be regarded as replacement therapy or that they influence the course of shock favorably through an influence on capillary permeability or tonus, we must rely on clinical observations or general experimental results.

Studies on patients, always restrictive, are particularly so in critical states of circulatory failure. The reactions of patients to so subtle a form of therapy as adrenal cortex extract are difficult to interpret owing to the uncertainty as to the course that would have been followed without therapy. Furthermore, other forms of treatment are usually given concurrently in order that a patient's chance of recovery may not be jeopardized. If the favorable clinical reports (e.g., 18, 19, 20, 21, 22, 23) are critically examined as to the evidence of efficacy rather than with regard to the conclusions voiced, few experimentalists would probably be convinced that real clinical benefit has been demonstrated. Furthermore, such impressions are at least partly contradicted by recent statistical studies (24, 25), and Robb (26) calls attention to possible deleterious cardiac effects of DCA.

A critical analysis of experimental work also leaves the impression that in many instances the conclusions as to the practical value of adrenal cortical preparations in shock were not always warranted. A few examples may be given. Perla and associates (27) showed that adrenal cortex preparations enhance the resistance of adrenalectomized rats to lethal doses of histamine. However, no evidence is at hand that such death was due to shock and some question exists as to whether histamine can produce shock. Moreover, the fact that injection of epinephrine also protects adrenalectomized rats against histamine (28) suggests that there is nothing specific about the effect of ACE.

Selye and his associates, stimulated by their hypothesis of a shock-countershock sequence to all forms of noxious agents, carried out several series of experiments on rats (for review cf. 29). These led to the conclusion that pure corticosterone and ACE are very effective in combatting shock caused by surgical trauma and other means, whereas DCA is ineffective. A review of the work makes us hesitate to accept so sweeping a conclusion. They produced shock in rats in two ways; (*1*) by injection of formalin and (*2*) by hemostatic crushing of the intestines. The number of animals used in any series was not large. They used changes in blood volume, chlorides, sugar and hemoglobin as a criterion of shock and for evaluation of treatment. Changes in blood chlorides gave the most consistent results. We cannot accept such a criterion of shock or judgments of therapy based on such evidence, particularly since in one report (30) the data presented in animals shocked by intestinal trauma were not statistically significant as regards effects of ACE, and in another report (29) corticosterone treated rats only showed significant difference in chlorides when shock was produced by formalin injection and in bleeding volume at death in animals shocked by intestinal trauma.

Employing a different method for producing standardized body trauma without hemorrhage in rats, Noble and Collip (31) in the same institution presented data of survivals from which no practical beneficial effect of ACE treatment could be deduced. Root and Mann (17) on the basis of survival and observation of capillaries during life, recently came to the same conclusion regarding the ineffectiveness of ACE in shock produced by ligation and release of a limb. In summary, it is our impression that experiments on rats are negative as regards the efficacy of ACE in shock.

Heuer and Andrus (32) produced shock in dogs by injections of aqueous extracts of contents from obstructed intestines. The extracts probably contained histamine but were not chemically analyzed. They report that the effectiveness of plasma infusions is materially enhanced by addition of ACE. The number of experiments upon which this conclusion was based is not stated and determinations of plasma volumes in the experiments presented were made only at infrequent intervals. In view of the comparatively weak solutions of ACE which were available at the time and the emphasis which others place on the use of large doses of highly potent extracts, these and similar experiments should be confirmed.

Wohl, Burns and Pfeiffer (33) report an apparent benefit and prolonged survival of dogs with acute intestinal obstruction when physiological salt solution and ACE were used. They appreciated that their few experiments permitted only probable and not very certain conclusions.

The observations of Weil, Rose and Browne (6) seem to suggest that ACE is effective and DCA ineffective in shock produced in rabbits by intestinal manipulation. In an adequate number of animals they found a mortality of 19% of ACE-treated as against 62% of untreated rabbits. In contrast to results with DCA, these differences are obviously significant statistically. The only question which arises is whether it is possible to inflict equivalent degrees of trauma by such a procedure. Without such assurance, any statistical analysis loses its meaning. One of us (W) has on previous occasions attempted to use this method on dogs and a survey of results during separate periods shows almost as wide statistical variations in different untreated groups of dogs.

The most substantial evidence has recently come from the Michael Reese laboratories. Perlow, Killian, Katz and Asher (34) devised a method of producing shock in dogs by fairly complete venous occlusion. Loss of blood plasma,—evidenced by swelling of the leg, histological sections, hematocrit changes and the prevention of shock by application of a plaster cast (35)—seemed to be the initiating factor, but not the sole factor determining irreversibility. The procedure seems to operate almost as effectively in anesthetized dogs (36).

In such dogs, pretreatment with 1 3 to 3 mgm./kilo DCA decreased the initial rate of swelling, though ultimately this equaled that of control animals. Nevertheless, the dogs appeared in better condition following venous occlusion and the percentage of recoveries increased greatly. Still more recently, Schleser and Asher (16) found, however, that while pre-treatment with ACE reduces the ultimate degree of swelling, the percentage of survivals is less than in pretreatment with DCA, but significantly greater than without treatment.

An attempt to account for such positive and our negative findings on the basis that we created different types of shock and used different criteria is not wholly satisfying. There are as many similarities as there are differences. In both methods for producing shock the process was inaugurated by a reduction in circulating volume, the visceral changes found at necropsy were similar (though perhaps differing in intensity) and the percentage of deaths constituted the real criterion. However, the cause of the irreversibility remained unrevealed to both of our groups and may have involved different mechanisms or organs. It is conceivable that the great loss of red corpuscles and stagnant anoxia in our animals accelerated the irreversible mechanisms to such an extent that they could not be halted by agents which are still effective when the damage develops more slowly.

Reflections such as these require us to conclude that while our experiments render it improbable that adrenal cortex preparations are beneficial in shock which follows prolonged post-hemorrhagic hypotension they should not be quoted as precluding their effectiveness in types of shock in which loss of blood corpuscles or preshock hypotension are not significant factors.

SUMMARY

1. Technical details are given regarding a method for producing irreversible hemorrhagic shock by a two-stage bleeding and production of successive 50 mm. and 30 mm. hypertensions for definite periods.

2. Using this technique for producing standardized hemorrhagic shock in a new series of 21 control dogs, 76.5% succumbed either from a precipitant or delayed circulatory failure after reinfusion of all the blood withdrawn.

3. Dogs pretreated and/or treated with ACE during the stage of moderate hypotension showed a 75% mortality. The incidence of a precipitant shock was greater and the pathological congestion and hemorrhagic tendency of the upper intestine and endocardium appeared to be more pronounced.

4. A study of consecutive changes in auricular and effective venous pressures showed no difference in treated and nontreated dogs. When, toward the end, circulatory failure had supervened, effective venous pressures were sometimes above and sometimes below starting pressures in untreated animals but generally below original pressures in dogs treated with ACE. Development of precipitant shock or maintenance of an efficient circulation were not related to the trends of venous pressure.

5. On the basis of the incidence of irreversible circulatory failure, the course in its development, changes in venous pressures and pathological appearances at necropsy, we were unable to detect any beneficial effects from pretreatment or treatment of dogs with large doses of ACE in hemorrhagic shock.

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THE ESTIMATION OF QUININE IN HUMAN PLASMA WITH A NOTE ON THE ESTIMATION OF QUINIDINE[1]

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Chemotherapeutic studies of malaria have demonstrated the desirability of a simple method for the routine estimation of the concentration of quinine in human plasma. Methods are available for the estimation of quinine in the blood but the majority of these involve the preliminary separation of the quinine by serial extractions and its concentration in a sufficiently small final volume for assay. Such methods have a high degree of specificity but are time-consuming.

The present method obviates such extractions by precipitating the proteins in aqueous solution with metaphosphoric acid at a high dilution. The quinine in the filtrate is then directly estimated in the filtrate through its fluorescence in ultraviolet light. The measurement of quinine concentration by such a method includes fluorescent degradation products of the quinine if such are present. The error due to this circumstance is small and quite constant in the case of human plasmas. This fact, together with the simplicity and precision of the procedure, recommends the method for clinical use in the control and evaluation of quinine therapy in the human subject.

EXPERIMENTAL. *Protein Precipitation.* A difficulty in the determination of quinine in simply prepared protein-free filtrates of plasma arises from the tendency of quinine to be adsorbed on or occluded by the protein precipitate. This loss is determined largely by the precipitant used and to a lesser extent by the dilution at which the precipitation is performed. The most suitable precipitant of those tried is metaphosphoric acid. Loss of quinine during the precipitation of the plasma proteins is completely avoided with this precipitant by performing the precipitation at a high dilution and by the use of adequate amounts of acid.

Assay of Quinine. The fluorometer selected for use in the present method is the Coleman Photofluorometer. This selection was based on the high sensitivity of the instrument, the linear relationship which obtains between galvanometer deflection and quinine concentration over the desired range (0.01 to 0.3 micrograms per cc.) and the speed and simplicity of operation. The latter are desirable features and result largely from the design of the optical system which permits the use of selected 18 mm. test tubes instead of the conventional cuvettes.

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

Quinine in acid solution fluoresces strongly in ultraviolet light, and the intensity of emitted light is proportional to the quinine concentration when the latter is low. These circumstances facilitate the use of fluorescence in the estimation of quinine concentration. There are substances in plasma filtrates which quench fluorescence. This phenomenon exists as a difficulty in the direct determination of quinine in plasma filtrates. However, it does not change the linear relationship of the intensity of fluorescence to quinine concentration and so introduces no error in the estimation provided the quinine standards are prepared using quinine free plasma filtrates as the diluent.[2]

Properly prepared filtrates of plasma manifest a small amount of fluorescence. This is chiefly due to the native fluorescence of the glass cuvette and, to a smaller extent, to the reagents used in the precipitation. Non specific fluorescence of the filtrates is negligible and may be disregarded.

REAGENTS. *Standard Quinine 100 mgm. per liter.* 120.7 mgm. of quinine sulfate $(C_{20}H_{24}O_2N_2)_2 \cdot H_2SO_4 \cdot 2H_2O$ are dissolved in tenth normal sulfuric acid and made up to 1 liter. It is stored in the ice box in an amber colored bottle. The solution is stable under these conditions. From this solution working standards are prepared by dilution with water. A solution of 1 mgm. per liter retains its strength for at least a week with no other precautions than storage in the ice box.

Metaphosphoric Acid 20%. The acid sticks are dissolved by continuous shaking without heating. Since this solution slowly undergoes hydrolysis to phosphoric acid, it is made up fresh every two weeks and stored in the ice box.

PROCEDURE. *Plasma.* To a mixture of 1 part plasma and 39 of water 10 parts of metaphosphoric acid are added with vigorous shaking. After 15 minutes the mixture is centrifuged at high speed for 10 minutes. The clear supernatant liquid is decanted into a cuvette and compared in the photofluorometer with a suitable standard. No acid is added since the filtrate is sufficiently acid to produce maximal fluorescence.

Estimation of Quinine in Plasma Filtrates. The standard is made up of filtrate of a quinine free plasma sample prepared in the usual manner. It is prepared by the addition of 0.5 cc. standard quinine solution to 10 cc. of the protein free filtrate, which can be conveniently done in a cuvette. A further portion of the quinine free filtrate is used for the blank setting of the instrument. The 5% difference in the concentration of quenching substances in the unknown and standard introduces no measurable error.

The Coleman glass filter B_1S (Corning 587) is used to isolate the activating energy if the concentration of quinine in the filtrates to be analyzed is less than 0.08 mgm. per liter; the Coleman B_1 filter is used for high concentrations.[3] A

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[2] The fluorescence of a plasma filtrate containing quinine appears to differ from that of an aqueous solution mainly insofar as the filtrate contains chloride. A 17% quenching of fluorescence results from this circumstance and is quite constant from one filtrate to another.

[3] Same as B_1S filter with the addition of a neutral screen and thus transmitting only 28 per cent of the activating light.

Coleman glass filter PC_1 (filter combination = Corning 038+ 428) is used to transmit the fluorescent light.

Calculation of Results. The calculation of the quinine concentration of the filtrate is by simple proportion. In a 1:50 plasma dilution, using a 1 mgm. per liter quinine standard

$\frac{x}{y}$ times $\frac{0.5}{10.5}$ times 50 = mgm. of quinine per liter of original sample where

x = reading of unknown

y = reading of standard

TABLE 1

Recovery of quinine from 1 cc. of plasma

QUININE ADDED	PLASMA 1:50 ppt.	
	Quinine found	Recovery
µgm.	*µgm.*	*per cent*
0.5	0.46	92
	0.52	104
	0.47	94
	0.49	98
1	0.99	99
	1.00	100
	0.94	94
	1.04	104
2	2.02	101
	2.04	102
	2.03	101
	2.08	104
3	3.03	101
	2.99	100
	3.04	101
	3.07	102
5	4.90	98
	5.08	102
	5.00	100
	4.84	97

The concentration of quinine in the original sample (mgm./liter) is equal to the product of the galvanometer reading and 0.0397 when the precipitation dilution is 1:50 and the instrument is set at a galvanometer reading of 60 with the above standard.

Precision. Recoveries of quinine added in various amounts to human plasma were performed to test the accuracy of the method. A series of such recoveries are presented in table 1.

These are consecutive analyses and are representative of other similar series

TABLE 2

Reproducibility of the estimation of quinine in plasma

TISSUE	DAY OF ANALYSIS	QUININE
		mgm per liter
Plasma A	1	3.07
		3.00
	2	2.98
		2.98
Plasma B	1	2.20
		2.26
		2.20
		2.22
		2.20
		2.20
	2	2.25
		2.24
	4	2.19
		2.29
		2.19
		2.19

TABLE 3

Comparison of results obtained by extraction and protein precipitation procedures

SPECIMEN	EXTRACTION PROCEDURE A	PROTEIN PRECIPITATION PROCEDURE B	RATIO A/B
	mgm/liter	*mgm/liter*	
1	1.64	1.94	0.85
2	2.70	2.99	0.90
3	5.52	6.21	0.89
4	5.46	6.43	0.86
5	7.08	7.84	0.90
6	3.53	3.78	0.94
7	3.64	4.28	0.85
8	1.17	1.25	0.94
9	5.46	5.98	0.91
10	7.23	8.52	0.85
11	8.40	9.01	0.93
12	6.90	7.01	0.98
13	5.29	6.13	0.87
14	6.80	7.33	0.93
15	0.95	0.98	0.97

performed over a period of a year. The stability of the quinine in plasma and, incidentally, the reproducibility of the estimation is demonstrated by the data in table 2.

The latter analyses, performed immediately and on succeeding days, were on samples of plasma of patients to whom quinine had been administered *per os.*

Specificity. A series of human plasma samples were studied to determine the extent to which the metaphosphoric acid filtrates contain fluorescent degradation products of quinine with different solubility characteristics. Fifteen plasma samples obtained from 8 patients on quinine therapy were used in the examination. Plasma quinine concentration was estimated by the method detailed above and by an extraction procedure. The latter (1) involves an extraction of the quinine into ether as the free base and its return to an aqueous phase as the salt before fluorometric assay. This examination (table 3) indicates that the present simplified procedure in the estimation of concentration includes a small and fairly constant proportion of fluorescent degradation products which have solubility characteristics different from quinine. This amounts to a mean of 11%. The maximal variation in the case of individual samples was 2 and 15%.

DISCUSSION. It is clear that recovery of quinine from plasma is excellent at concentrations of 1.0 mgm. per liter and higher. The method as described has precision and speed. These characteristics together with a reasonable degree of specificity recommend it during quinine therapy of malaria. The reproducibility of the analysis of plasma samples on succeeding days demonstrates that quinine in these fluids is quite stable, so that samples may be stored a number of days prior to analysis.

It is of interest to note that of the other important alkaloids of cinchona bark, only quinidine was found to fluoresce appreciably. The intensity of fluorescence of quinidine is the same as quinine and a limited series of recoveries from plasma yielded results comparable to those reported here for quinine.

SUMMARY

A simple precise method is described for the estimation of quinine in human plasma. The proteins of these fluids are precipitated with metaphosphoric acid and the quinine concentration in the filtrate assayed by measuring the intensity of its fluorescence in ultraviolet light. An identical procedure may be used for the estimation of quinidine in plasma.

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THE COMPARATIVE ACTIVITY OF QUININE, QUINIDINE, CINCHONINE, CINCHONIDINE AND QUINOIDINE AGAINST PLASMODIUM LOPHURAE INFECTIONS OF PEKIN DUCKLINGS

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The present shortage of quinine has focused attention on totaquine, a mixture of the cinchona alkaloids, as a substitute for quinine in the control of malaria. The original standards (1) for totaquine, which were established by the Malaria Commission of the League of Nations, specified that the mixture should contain no less than 15% of quinine. The following monograph will appear in a supplement to U.S P. XII: "Totaquine is a mixture of alkaloids from the bark of *Cinchona succiruba Pavon* and other suitable species of Cinchona. It contains not less than 7% and not more than 12% of anhydrous quinine and a total of not less than 70% and not more than 80% of the anhydrous crystallizable cinchona alkaloids, the designation crystallizable alkaloids referring to cinchonidine, cinchonine, quinidine and quinine". In order to decide what effect a lowering of the quinine content and change in proportion of the other crystallizable alkaloids might have on the antimalarial activity of the final product, it is essential that we know the relative activity of the cinchona alkaloids. It is, furthermore, essential that the toxicity of the alkaloids be known otherwise variations in the constitution of totaquine might be accompanied by serious alterations in the toxicity of the drug.

The literature contains a number of reports concerning the activity of the cinchona alkaloids against avian malaria Sergent, Sergent and Catanei (2) found cinchonidine to be slightly less active than quinine in retarding the appearance of parasites in infected canaries and cinchonine in turn to be less active than cinchonidine. They did not investigate the activity of quinidine as they regarded it to be too toxic to deserve consideration. Giemsa, Weise and Tropp (3) however, reported that quinine and quinidine were about equally active against canary malaria and that cinchonine was the least active of the three On the other hand, Goodson, Henry and Macfie (4) state that in their experiments there was little difference in activity among the four alkaloids. Buttle, Henry and Trevan (5), in their studies on canary malaria, found cinchonidine and quinidine to be about one half as active and cinchonine less than one fifth as active as quinine.

Although the published data do indicate that all four crystalline alkaloids show antimalarial activity, it is apparent that the various investigators do not agree on the relative activity of the drugs. The experiments cited above were all performed on canary malaria which until recently was the most satisfactory available means for studying the chemotherapy of malaria in the laboratory. The relative activity of the drugs was estimated by comparing the average incubation period of the treated with that of untreated controls. Unfortunately there was a wide variation in the time of appearance of the parasites following intramuscular inoculation of infected material, Goodson *et al.* (4) stating that in their experiments the incubation period varied from three to eleven days with parasites appearing between six and nine days in 83% of their birds. It is probable that in none of the studies mentioned was a sufficiently large number of canaries used so that a satisfactory quantitative comparison of the alkaloids could be made.

The recent discovery by Wolfson (6) that ducks could be infected with *Plasmodium lophurae* has given experimental chemotherapy of malaria a valuable tool. This parasite responds to the administration of drugs which are effective in human malaria. Studies have been published on the effect of drugs on *P. lophurae* infections of ducklings (7, 8). It has been found, furthermore, that the response of the infections to given doses of the drug is quite constant, provided that uniform experimental conditions are maintained, and this offers a method for a reasonably quantitative comparison of the activity of antimalarial drugs. In this communication we report experiments which compare the activity of quinine, quinidine, cinchonine, and cinchonidine against the schizonts of *Plasmodium lophurae*, together with data on the activity of two samples of totaquine and of a sample of quinoidine, the name given the residue following the extraction of the crystalline alkaloids.

EXPERIMENTAL. Pekin ducklings weighing about 50 grams each were inoculated intravenously with 2,000,000 erythrocytes parasitized by *P. lophurae*. The course of the infections was followed by daily parasite counts beginning on the fourth day following inoculation. In making the parasite counts, 10,000 red cells were counted. In untreated birds the course of the disease was acute with an average of 80% of cells parasitized on the 10th day and death of the majority of the birds on the 10–12th day. The Quinine Sulfate U.S.P., Quinidine Sulfate U.S.P., Cinchonine Sulfate N.F., and Cinchonidine Sulfate N.F. which were used in these experiments were obtained from a commercial source and were not submitted to further purification. The quinoidine sulfate represented the residue of a lot of bark after the extraction of crystalline alkaloids. This material varies widely from lot to lot and its chemistry is not understood. The analysis[1] of the two samples of totaquine was as follows:

	TOTAQUINE I	TOTAQUINE II
	per cent	*per cent*
Anhydrous quinine	18.9	55.6
Anhydrous cinchonidine	28.9	24.6
Anhydrous cinchonine	32.0	4.7
Anhydrous quinidine	0.0	0.0
Total alkaloids	79.8	84.9

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[1] The analysis of these samples was given us by Dr. Rosin of Merck & Co., Inc.

The drugs were dissolved in water and given to the ducklings by stomach tube once daily for the first six days following inoculation, with daily adjustment of dose to the weight of the duck. The experimental results are tabulated in table 1. For convenience the daily parasite counts on each group of birds have been averaged. It is apparent that there is relatively little difference in activity among quinine, quinidine, cinchonine and cinchonidine. Both samples of totaquine showed activity comparable to that of quinine. The quinoidine was considerably less active than the crystalline alkaloids.

TABLE 1

Activity of cinchona alkaloids against the schizonts of Plasmodium lophurae in Pekin ducklings

DRUG*	DOSE	NO OF DUCKS	NO OF PARASITIZED ERYTHROCYTES PER 10,000 ERYTHROCYTES DAYS AFTER INOCULATION						
			4	5	6	7	8	10	12
	mgm per kgm								
Cinchonidine	10	4	29	84	92	264	286	1440	2700
	20	12	6	7	4	3	2	1	4
	40	4	2	4	2	0	3	1	0
Quinidine	10	4	33	89	95	141	213	923	1972
	20	12	10	9	5	4	1	1	2
	40	4	3	4	4	3	1	2	1
Cinchonine	10	4	32	55	88	116	107	433	920
	20	12	17	20	18	18	19	40	330
	40	4	4	7	6	2	3	1	2
Quinine	10	4	30	47	69	108	527	5291	4667
	20	12	10	14	13	10	5	23	146
	40	4	1	2	3	0	3	0	0
Quinoidine	100	3	32	48	88	153	208	1025	2744
	200	3	13	17	25	35	76	107	677
	300	3†	6	6	11	5	8	11	32
Totaquine I	20	8	13	12	13	9	7	3	93
Totaquine II	20	8	2	3	3	2	1	0	7
Controls		20	65	153	520	1155	3363	7652	Dead

* Drugs were administered in aqueous solution by stomach tube once daily for the first six days following inoculation.

† One duck died on the 5th day

Toxicity determinations were made on 20 gram albino mice of the Carworth Farms CFI strain These mice had been kept in an air-conditioned laboratory for three weeks on a stock diet and appeared to be in good condition when placed on experiment. The drugs in aqueous solution were administered by stomach tube in one dose and the mice were kept under observation for seven days. The results of these experiments are given in table 2. The acute oral toxicity of the four crystalline alkaloids in the mouse is about the same, although cinchonine is slightly more toxic than the other three. These toxicity figures are about the same as those reported by Buttle, Henry and Trevan (5) for canaries.

TABLE 2

Oral toxicity of cinchona alkaloids in mice

SAMPLE	DOSE	NO. OF ANIMALS	MORTALITY 24 HOURS	LATE DEATHS: DAYS					
				2	3	4	5	6	7
	grams/kgm.								
Quinoidine	0.4	10	3	0	0	0	0	0	0
	0.6	10	8	0	0	0	0	0	0
	0.8	10	8	0	0	0	0	0	0
	1.0	10	10						
	1.2	10	10						
Cinchonine	0.6	10	0	0	0	0	0	0	0
	0.8	10	3	0	0	0	0	0	0
	1.0	10	6	0	0	0	0	0	0
	1.2	10	7	0	0	0	0	0	0
	1.4	10	8	0	0	0	0	0	0
	1.6	10	8	0	0	0	0	0	0
Quinidine	0.4	10	0	0	0	0	0	0	0
	0.6	10	1	0	0	0	0	0	0
	0.8	10	4	0	0	0	0	0	0
	1.0	10	9	1					
	1.2	10	9	0	0	0	0	0	0
	1.4	10	9	0	0	0	0	0	0
Quinine	0.6	10	0	0	0	0	0	0	0
	0.8	10	4	0	0	0	0	0	0
	1.0	10	5	0	0	0	0	0	0
	1.2	10	8	0	0	0	0	0	0
	1.4	10	7	0	0	0	0	0	0
	1.6	10	8	0	0	0	0	0	0
Cinchonidine	0.4	10	1	0	0	0	0	0	0
	0.6	10	4	0	0	0	0	0	0
	0.8	20	11	0	0	0	0	0	0
	1.0	10	8	0	0	0	0	0	0
	1.2	10	10	0	0	0	0	0	0

The fact that there is little difference in the activity of the four crystallizable cinchona alkaloids against the schizonts of *P. lophurae* and that the acute oral toxicity of the four drugs in the mouse and the canary is about the same suggests that a mixture of the alkaloids should prove as satisfactory an antimalarial as quinine regardless of the relative proportion of the individual drugs in the mixture. Quinoidine should not be used as it is less active and more toxic than the crystallizable alkaloids.

SUMMARY

1. Quinine, quinidine, cinchonine and cinchonidine show about the same activity against the schizonts of *P. lophurae* in Pekin ducklings.

2. Quinoidine is considerably less active than the four crystalline cinchona alkaloids.

3. Two samples of totaquine varying widely in their quinine content were as active as quinine in duck malaria.

4. There is little difference in the acute oral toxicity for mice among the four crystalline alkaloids. Quinoidine is more toxic than the crystalline alkaloids.

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FLAVICIN: AN ANTIBACTERIAL SUBSTANCE PRODUCED BY AN ASPERGILLUS FLAVUS[1]

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Since the discovery of *penicillin* by Fleming (1) in 1929 a number of investigations have been carried out on antibacterial substances produced by microorganisms. As a result of these investigations it has been shown during the past few years (2–7) that besides Fleming's *Penicillium notatum* other fungi and bacteria are able to produce powerful bacteriostatic and bactericidal agents. Some of these substances such as *gramicidin* and *tyrocidin* (8) are quite toxic. On the other hand, *penicillin* (9) seems to have almost no toxic action. The search for relatively non-toxic substances among this group of extremely active bacterial inhibitors is of great interest for the development of new chemotherapeutic agents.

While studying the production of *penicillin* we isolated a mold belonging to the *Aspergillus flavus* group which produced a powerful antibacterial substance. This mold appeared as a contaminant on one of our cultures of *Penicillium notatum* and our interest was aroused in it due to our finding that it was able to dissolve *Staphylococcus aureus* and *Staphylococcus albus*. On further study we have found that this Aspergillus released in the culture medium an inhibitory substance which was active against a large number of bacterial species. We have succeeded in partially purifying this substance to an extent that its activity per milligram is comparable to that of "therapeutic" *penicillin* (9, 10) against Gram positive cocci.

Reviewing the literature we were able to find only two references to antibacterial substances produced by molds of the *Aspergillus flavus* group. White (3) reported in 1940 that a strain tentatively identified as belonging to the *Aspergillus flavus* group released in its cultures substances which inhibited the growth of a number of bacteria. In a further communication White (4) mentions a crystalline substance extracted from cultures of an *Aspergillus flavus* which inhibited the growth of *Streptococcus hemolyticus* and other organisms. It is impossible to tell from the available data whether there is any relationship between the antibacterial substance obtained by White and ours.

This present paper describes the optimum conditions for growing our mold, the extraction and partial purification of the active substance, to which we shall refer as *flavicin*.[2] We shall also describe the activity of the substance against 17 bacterial species and its acute toxicity in mice.

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[1] The funds for carrying out this work were kindly given to us by the Mallinckrodt Chemical Works.

[2] In an abstract of this paper (11) our antibacterial substance is referred to as *aspergillin*. Due to the possible confusion with White's aspergillic acid (12) we thought it advisable to change the name of our antibacterial substance to *flavicin*.

EXPERIMENTAL. *Properties of the mold.* The mold isolated by us has the morphological and cultural characteristics of the yellow green Aspergilli described as the *Aspergillus flavus* group in Thom's monograph (13). Our strain grows well on ordinary media and the growth is especially rich on media to which dextrose or other sugars have been added. Under these conditions the mold covers the surface of the culture media in 24–28 hours at 35–37°C. The mold grows well at room temperature although it covers the surface somewhat more slowly. The color of the conidia varies from light yellow to dark green depending on the medium and on the age of the culture. When inoculated on a culture of *Staphylococcus aureus* or *Staphylococcus albus* the mold grows well on the surface and the Staphylococci are dissolved in 2–3 days at 37°C.

Production and testing of flavicin. For the production of *flavicin* the mold is grown by us on the following modified Czapek Dox medium.

$NaNO_3$	U.S.P.	3.0	Grams
Dextrose	Pract.	20.0	Grams
Na_2HPO_4 (anhydrous)	C.P.	2.0	Grams
$MgSO_4\cdot 7H_2O$	U.S.P.	0.25	Grams
Concentrated corn steep water[3]		20.0	cc.
Distilled water to make		1000.0	cc.

The medium is distributed in special culture flasks in shallow layers approximately 2 cm. deep. The antibacterial principle appears in appreciable quantities in four days and its concentration increases for 3–4 days. Cultures grown at 35°C. are ready to be harvested six to eight days following inoculation.

The activity of the culture fluids is tested by the serial dilution method in broth tubes against a strain of *Staphylococcus aureus*. The serial dilution method, although somewhat laborious, has been found by us quite reliable and easy to duplicate. Previous to testing the culture fluids, samples thereof are filtered through glass bacteria filters to insure sterility. Our *Staphylococcus aureus* strain has been dried by the lyophilic method and is maintained in the dried state. A 24 hour broth culture is used for the actual testing. To simplify our discussion we shall refer to the dilution factors as Units. If a given *flavicin* solution inhibits the growth of our test organism at a dilution of 1:1000 we refer to that solution as one having 1000 Units per cc. A Unit is then the amount of active substance which dissolved in 1 cc. of broth inhibits the growth of our strain of *Staphylococcus aureus* (standard inoculum approximately 40,000 organisms per cc.) for 24 hours. Our end point is a perfectly clear broth solution. Control cultures are made with every test.

Aspergillus cultures of 6–8 days have an activity of 10–30 Units per cubic centimeter. The medium has a considerable influence on the bacteriostatic activity produced by the mold. The addition of corn steep water to the modified Czapek Dox medium greatly increases the production of *flavicin*. However a careful study of the ideal conditions for *flavicin* production has not yet been made by us. Just as the production of *penicillin* has been made much more efficient since the first studies carried out by Fleming (1) we believe that the *flavicin* production could be increased by finding the ideal cultural conditions.

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[3] Supplied by the A. E. Staley Manufacturing Company, Decatur, Illinois.

Extraction of flavicin. The cultures were prepared for extraction by filtering through a fine sintered glass Buchner funnel with calcium carbonate as a filter aid. The clear filtrate was acidified in 200 cc. portions to pH 2.5–3 with 5M phosphoric acid and extracted by purified isopropyl ether in a spray column. By extraction of the ether with a slight excess of 0.2N $NaHCO_3$ (5–10 cc. per liter of culture) a yield of 75–100% of the active material was usually obtained.

TABLE 1

Activity of partially purified flavicin against 16 bacterial species*

BACTERIAL SPECIES	MGM OF FLAVICIN PER CC IN BROTH PRODUCING COMPLETE BACTERIOSTASIS FOR 24 HOURS
Staphylococcus aureus . .	.008 or 1:125000
Staphylococcus albus .	008 or 1:125000
Streptococcus hemolyticus	.0016 or 1:625000
Corynebacterium diphtheriae (Gravis type)	*.0032 or 1:312500*
Bacillus anthracis	.0016 or 1:625000
Eberthella typhosa	*.8 or 1:1250*
Salmonella paratyphi A	Not inhibited by .8 mgm./cc.
Salmonella paratyphi B	Not inhibited by .8 mgm./cc
Escherichia coli	Not inhibited by .8 mgm /cc.
Bacillus Friedländeri	Not inhibited by .8 mgm./cc.
Pasteurella pestis .	Not inhibited by .8 mgm /cc.
Shigella dysenteriae	.8 or 1:1250
Shigella paradysenteriae Flexner	8 or 1:1250
Brucella abortus	06 or 1:16600
Vibrio cholerae	8 or 1:1250
Bacillus subtilis	.16 or 1:6250

* The sample of partially purified *flavicin* used in this table was the one described above which weighed 290 mg.

Purification of flavicin. Partial purification of the *flavicin* extracts is accomplished by acidifying the sodium bicarbonate solution of *flavicin* and removing the precipitate formed by filtration. The filtrate contains most of the antibacterially active material while the precipitate contains most of the toxicity. In a typical experiment 50 cc. of a bicarbonate solution containing 800±200 Units per cc. was cooled in ice and acidified to pH 2–3 with 5M H_3PO_4. The yellow precipitate was removed by filtration and the aqueous solution shaken under CO_2 with 50 cc. of ice-cold isopropyl ether, previously saturated with CO_2. The isopropyl ether layer was pipetted off, washed under a CO_2 atmosphere with two small portions of cold distilled water, centrifuging each time. The aqueous layers were then likewise washed successively with a second 50 cc. portion of isopropyl ether. The combined isopropyl ether solutions were distilled at 0° to dryness in a stream of CO_2 at a pressure of 30–35 mm. Hg. The yellow-orange glassy residue weighed 290 mgm., was dissolved in 5 cc. of 0.4N sodium bicarbonate solution and used for the determination of the activity of partially purified *flavicin.*

Activity of flavicin. The activity of our partially purified solid material tested against 16 bacterial species can be seen in table 1. In a later experiment it was found that *Pneumococcus Type III* was also inhibited by 0.2 Staphylococcus Unit per cc. The inhibition of bacterial growth at the dilutions represented in the table is complete. Partial inhibition takes place at much higher dilutions; however partial inhibition is difficult to measure accurately and it has not been included in the table.

Toxicity of flavicin. The concentrated crude extracts of these Aspergillus cultures are quite toxic. By the purification procedure described above the toxicity of the extract can be decreased considerably. The median lethal dose of our partially purified *flavicin* (the sample used for table 1) was 40 mgm./kgm. (5000 Units/kgm.) when administered to mice by the intraperitoneal route. Further purification of the antibacterial substance has been accomplished after filtering off the toxic precipitate, by extracting the filtrate with several portions of toluene. Subsequent extraction by isopropyl ether yields a product which has an activity of 160 Units per mgm. and which failed to cause any effect in 10 mice when injected intraperitoneally in the amount of 375 mgm./kgm.[4]

Mode of action of flavicin. As indicated in table 1 *flavicin* inhibits the growth of a number of bacterial species at high dilutions. It was of interest to determine whether the antibacterial action of *flavicin* consists purely of inhibition of growth or whether it actually kills bacteria. In an experiment, represented in table 2, *flavicin* solution was added to a suspension of *Staphylococcus aureus*

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[4] After the submission of this manuscript for publication we obtained a sufficient amount of our purest *flavicin* to carry out a limited experiment on its activity *in vivo*, the results of which are shown in the following table. The amount of drug available made it necessary to treat relatively few mice with rather low doses for a limited period. At no time was there the slightest evidence of symptoms in the surviving animals which could be attributed either to the infection or to the action of *flavicin*.

Action of flavicin on pneumococcal infection in mice

Organism Pneumococcus Type I

Infection Intraperitoneal injection of 0.1 cc. of a dilution of a 12 hour broth culture

Treatment 15–30 minutes after infection *flavicin** was given intravenously (50 mgm./kgm.) and subcutaneously (50 mgm./kgm.), thereafter at three hour intervals 25 mgm./kgm. was given subcutaneously for a total of 51 hours (total dose 525 mgm./kgm. = 84,000 Units/kgm.)

DRUG	NUMBER OF MICE	CULTURE DILUTION	TOTAL NUMBER OF MICE DEAD AFTER							
			1 day	1.5 days	2 days	2.5 days	3 days	4 days	5 days	20 days
Flavicin	10	10^{-2}	0	0	0	0	1	1	2	2
None	20	10^{-2}	5	17	18	20				
None	10	10^{-6}	0	2	9	10				

* *Flavicin* as sodium salt in water 800 Units/ccm. = 5.0 mgm./ccm.

in broth in the amount of 30 Units per cubic centimeter. Samples were taken every hour and the number of viable bacteria was determined by plate counts. As shown in table 2, the number of viable organisms decreased considerably after one hour at 37° and after 3 hours more than 99.9% of the organisms were non-viable. We may conclude from this experiment that *flavicin* has a definite bactericidal action against *Staphylococcus aureus.*

Discussion. *Flavicin,* like *penicillin,* is a water-soluble and ether-soluble organic acid which has a powerful antibacterial activity. It thus differs from *gramicidin, tyrocidin* and *penicillin B* (14) which are protein or polypeptide in nature. *Flavicin* resembles *penicillin* in another respect: it is unstable in an acid environment, particularly on shaking with air. Although both *penicillin* and *flavicin* inhibit especially Gram positive cocci, on the basis of their activity against Staphylococcus aureus as a standard *flavicin* is more active against *C. diphtheriae, B. anthracis, Staphylococcus albus* and *Brucella abortus* than is *penicillin.* Although there is much work to be done in order to determine the range of activity of *flavicin,* from the available evidence we may conclude that it is a powerful antibacterial agent which affects a wide variety of bacterial species.

TABLE 2

Bactericidal action of flavicin against Staphylococcus aureus

TIME	NUMBER OF VIABLE ORGANISMS (THOUSANDS PER CC.)		
	Flavicin, 30 Units per cc.	Flavicin, 5 Units per cc.	Control broth
hours			
0	1351	2387	1987
1	800	1393	1525
2	25	218	
3	<4.2	53	26250
4	<.84	26	
5	<.17	16	
6	<.17	6	293750
24		<.005	

SUMMARY

A mold of the Aspergillus flavus group was found to produce an antibacterial agent. The extraction, partial purification, bacteriostatic and bactericidal activity and acute toxicity of this substance are described.

We wish to thank Dr. Roy C. Avery for supplying us with the strains of bacteria used in our experiments.

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A POSSIBLE METHOD FOR THE DETERMINATION OF PROLONGED ACTION OF BARBITURATES

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In demonstrating strychnine convulsions to a class for want of other rats some rats were used which had received sodium pentobarbital 32 mgm./kgm. about 22 hours before. Very few typical convulsions were obtained and none of the rats died. Therefore, it was decided to investigate the problem further. Other methods have shown the persistence of barbiturates in the body for some time, and it was thought this study might throw additional light on the problem.

Chemical analyses have revealed the presence of various barbiturates in the body many hours after their administration. Pentobarbital was found in traces in the blood of the dog 12 hours after the intravenous administration of 100 mgm./kgm. None was found in the blood, brain, kidney, liver, or muscle of a rabbit five and one-half hours after administration of the same dose (1). Barbital was found in dog blood 24 hours after the administration of sodium barbital 225 mgm./kgm. (2). Newborn rabbits contained barbital when delivered 18 hours after the mother received 100 mgm./kgm. of sodium barbital. There was none found in newborn rabbits delivered 42 hours after the administration of 250 mgm./kgm. of sodium barbital to the mother (3). Mezey (4) made some motor and computing tests on human adults twelve hours after the oral administration of dial 0.2 grams, barbital 0.3 grams, and phenobarbital 0.3 grams. The motor tests did not vary from normal but the performance of mathematical tests was decreased in all of 7 subjects who received phenobarbital, decreased in 4 of 7 by dial and equal in 3. After barbital 4 of 6 subjects showed unchanged performance, decreased in one and increased in one.

Procedure. Rats weighing 180 grams or more were divided into 4 main groups for study. Group I was used for investigations on strychnine and sodium pentobarbital, Group II for investigations on strychnine and sodium phenobarbital. Group III was used for investigations on picrotoxin and sodium pentobarbital and Group IV for investigations on picrotoxin and sodium phenobarbital. Within each of these groups one sub-group was given a barbiturate 20 minutes before the convulsant and a second sub-group was given a barbiturate 22 hours before the convulsant. The sub-groups within each group were matched for sex and weight. These were also matched against controls.

All drugs were given by intraperitoneal injection. Strychnine was given in doses of 3 mgm./kgm. as the sulfate. Picrotoxin was given in doses of 7 mgm./kgm. Sodium pentobarbital was given in doses of 32 mgm./kgm. This dose had been shown to produce a loss of righting reflex in 19 out of 20 rats (5). Sodium phenobarbital was given in doses of 19 mgm./kgm. This dose did not cause a loss of righting reflex in any rats studied. It produced varying degrees of inco-ordination in some of the rats and no observable effect in others. The relatively small dose of phenobarbital was chosen because of its known prolonged action.

Results and discussion The average weight of the rats before receiving either barbiturate and the average weight 22 hours later were the same Strychnine when given alone in the dose of 3 mgm /kgm killed 31 out of 40 rats (table 1) Of 40 rats receiving sodium pentobarbital 20 minutes before strychnine 7 died Of 40 rats which received the same barbiturate 22 hours before strychnine 21 died The difference in death rate between the rats receiving strychnine alone and those receiving sodium pentobarbital 22 hours before strychnine was 2 35 times the standard error of the difference This makes it seem probable that the difference is a real one The difference in death rate between the rats receiving strychnine 20 minutes after sodium pentobarbital and those receiving the convulsant 22 hours after the barbiturate is 3 48 times the standard error of the difference Although a greater number of rats are killed by strychnine 22 hours after sodium pentobarbital than 20 minutes after sodium pentobarbital, the pentobarbital seems to offer some real protection even 22 hours after its administration

TABLE 1

Effects of the administration of strychnine 3 mgm /kgm with and without barbiturates

	STRYCHNINE ALONE 3 MGM /KGM	Na PENTOBARBITAL 32 MGM /KGM		Na PHENOBARBITAL, 19 MGM /KGM	
		20 min before strychnine 3 mgm /kgm	22 hrs before strychnine, 3 mgm /kgm	20 min before strychnine 3 mgm /kgm	22 hrs before strychnine 3 mgm /kgm
No of male rats used	20	20	20	21	21
No of female rats used	20	20	20	11	11
No of rats used	40	40	40	32	32
No of rats dead	31	7	21	15	7
% of rats dead	77	17 5	52 5	46 9	21 9
S E %	6 7	6 0	7 9	8 8	7 8
Mean time of death (min)	*13 3*	*44 0*	*10 6*	*16 9*	*8 3*

Results on sodium phenobarbital (table 1) indicate protection both at 20 minutes and at 22 hours before strychnine It will be noted also that the protection is greater at 22 hours than at 20 minutes The difference between the two times of administration of sodium phenobarbital is 2 05 times the standard error of the difference In comparing these results with those of sodium pentobarbital it must be remembered that the dose of sodium phenobarbital was actually smaller, smaller relative to molecular weight, and markedly less in observable physiological effects

The studies on picrotoxin were carried out for comparison with the strychnine to determine if another convulsant would give similar results Picrotoxin was selected for two reasons (*1*) it has a different site of action than strychnine, (*2*) it is an antidote to the barbiturates as well as the reverse The dose of picrotoxin 7 mgm /kgm killed 19 of 20 female rats (table 2) When the dose of picrotoxin was preceded by sodium pentobarbital by 20 minutes 5 of 20 were killed, and when the interval was 22 hours 9 of 20 were killed. The difference between the two standard errors of the difference of the groups receiving sodium pentobarbital was only 1.36 times the standard error of the difference. The difference between the group treated with sodium pentobarbital 22 hours before picrotoxin and the group receiving picrotoxin alone was 4.13 times the standard error of the difference. The difference between the group receiving sodium pentobarbital 20 minutes before picrotoxin and the group receiving picrotoxin alone was 6.48 times the standard error of the difference. It therefore seems highly probable that the sodium pentobarbital provided good protection in both instances. The probability is between one in five and one in ten that the protection at 20 minutes was not greater than that at 22 hours after sodium pentobarbital.

For the work on sodium phenobarbital large male rats were used and it was necessary to have another set of controls. Of those receiving picrotoxin alone 11 of 20 died, of those receiving sodium phenobarbital 20 minutes before picrotoxin 3 of 20 died, and of those receiving the barbiturate 22 hours before picrotoxin 2 of 20 died. The chances are exceedingly small that there is any difference in the two groups receiving the sodium phenobarbital. The chances are very great, however, that good protection is provided by sodium phenobarbital both at 20 minutes and at 22 hours. The difference between the death rates with picrotoxin alone and with picrotoxin given 22 hours after sodium phenobarbital was 3.46 times the standard error of the difference. The difference between the death rates with picrotoxin alone and with picrotoxin given 20 minutes after sodium phenobarbital was 2.92 times the standard error of the difference.

TABLE 2

Effects of the administration of picrotoxin 7 mgm./kgm. with and without barbiturates

	PICROTOXIN ALONE, 7 MGM./KGM.	Na PENTOBARBITAL, 32 MGM./KGM.		PICROTOXIN ALONE, 7 MGM./KGM.	Na PHENOBARBITAL, 19 MGM./KGM.	
		20 min. before picrotoxin, 7 mgm./kgm.	22 hrs. before picrotoxin, 7 mgm./kgm.		20 min. before picrotoxin, 7 mgm./kgm.	22 hrs. before picrotoxin, 7 mgm./kgm.
No. of male rats used.				20	20	20
No. of female rats used....	20	20	20			
No. of rats dead..........	19	5	9	11	3	2
% of rats dead...........	95	25	45	55	15	10
S.E. %..................	4.9	9.7	11.1	11.1	8.0	6.7
Time range of death (min.)	11–42	51–180+	25–36	16–55	16–32	26–62
Mean time of death (min.)	28	125+	32	30	25	

The length of life of those rats which died is recorded in tables 1 and 2. The survival time was greater than the survival time of the controls when either convulsant was given 20 minutes after sodium pentobarbital. The length of life was not appreciably altered by sodium pentobarbital given 22 hours before either convulsant. Sodium phenobarbital did not seem to alter the length of life of those which died. The effect on survival time does not seem then to be as persistent as the effect on death rate.

The results from both strychnine and picrotoxin indicate a long persistent action of both sodium pentobarbital and sodium phenobarbital. This protective action against death from convulsants is present long after all other observable effects have worn off. The mechanism is as yet not understood. With such small doses it seems unlikely that much of the unchanged barbiturate is present (1, 2, 3). There may be active decomposition products in addition to a small amount persisting sufficient to account for the effects or there may be persistence of a physiological change. It was thought that this might be developed into a method for the study of length of action of barbiturates.

SUMMARY

(1) Sodium pentobarbital was effective against deaths from strychnine and picrotoxin hours after the signs of narcosis are no longer present.

(2) Sodium phenobarbital was more effective against deaths from strychnine when given 22 hours before the strychnine than when given 20 minutes before strychnine.

(3) Sodium phenobarbital was equally effective against deaths from picrotoxin whether given 20 minutes or 22 hours before picrotoxin.

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FURTHER STUDIES ON THE SEPARATION FROM KIDNEY TISSUE OF A SUBSTANCE CAPABLE OF REDUCING THE BLOOD PRESSURE IN EXPERIMENTALLY INDUCED HYPERTENSION[1]

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Since our first report (1) on the preparation of extracts derived from fresh kidney substance, several papers (2, 3) have appeared describing results obtained with renal extracts prepared by various procedures. It has not been clear in the reports of the different authors as to whether or not the effects observed were due to the same effective agent. Moreover, recent studies (4, 5) have demonstrated the ease with which non-specific depressor effects are capable of reducing the blood pressure of hypertensive animals or man. It is thus important to exclude these agents as the effective substances in evaluating the significance of the drop in blood pressure observed following the use of renal extracts.

Since our earlier report (1) we have devoted our attention to purification and concentration of the renal extract to learn something of its chemical nature and to determine if the observed results were due to the presence of a physiologically significant factor normally elaborated by the kidney. The conclusions presented in this paper are based on observations over a period of five years, involving several thousands of assays and more than 50,000 determinations of blood pressure on over a thousand animals. A typical assay is reproduced in figure 1.

MATERIALS AND METHODS. The crude extracts, prepared and supplied to us by the Eli Lilly Company, Indianapolis, were used in the present study. These consisted of dehydrated renal tissue or were prepared by precipitating an acid extract of pig's kidneys with ammonium sulfate as previously described (1). Dehydrated kidney tissue is much more convenient than the fresh glands as a starting material for preparing extracts, for it is possible to extract the active principle by distilled water without the addition of acid as in the case of fresh glands. The use of water reduces the amount of extraneous protein material dissolved in the initial extraction and results in a more concentrated and purer product in the preliminary stages of preparation. The aqueous extract is separated from the dehydrated tissue after standing with occasional shaking for at least 24 hours, filtered on a Buchner funnel, and precipitated by saturation with ammonium sulfate.

We have utilized the rat, rendered hypertensive by the application of cotton cloth to the kidneys (6), for assay purposes. The blood pressures were determined by the plethysmographic procedure (7) on the unanesthetized animal. The use of the rat offers an advantage over the dog in studies involving chemical fractionation, for by using five animals for each assay the inevitable biological variation is reduced to a minimum.

Except where indicated, the extracts were administered orally by admixture with food. In this way the effects induced by pyrogens, protein impurities or other non-specific contaminants which are known to lower blood pressure are excluded. However, even in the

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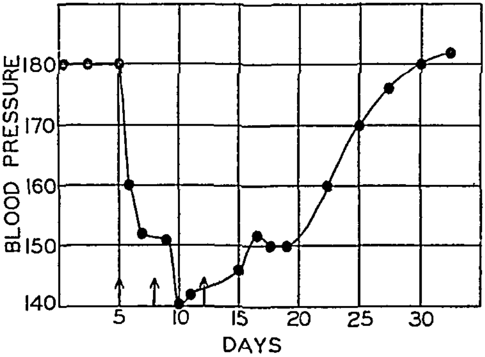
[1] Aided by grants from the John and Mary R. Markle Foundation, Mr. Joe Werthan and the National Life and Accident Insurance Company.

case of oral administration several sources of error may vitiate the results and must be avoided. Starvation, for example, will reduce the blood pressure of hypertensive rats, such effects being noted after 24 hours deprivation from food, and is very marked after 48 to 72 hours (4). When fed distasteful mixtures rats will reduce their food intake and undergo a drop in blood pressure. To detect this source of error we have routinely weighed our animals daily and any such assays in which an appreciable loss of weight occurred have been discarded.

The removal of excess ammonium sulfate. In laboratory preparations on a small scale it is possible, as described previously (1), to eliminate most of the ammonium sulfate present in the crude precipitate by washing on the filter. However, in preparations obtained by the usual industrial methods, or where it is desired to concentrate the activity further, it is necessary to resort to other procedures. Dialysis, the conventional procedure for accomplishing this, cannot be utilized as described later The addition of water to the ammonium sulfate-rich precipitate results in the solution of salts and if the amount of water be limited so that the concentration of ammonium sulfate is not less than 50% saturation, none of the active principle is dissolved. The suspension thus obtained is filtered and the filtrate discarded. The remaining precipitate is washed on the filter with a small *amount of water, as previously suggested (1), to remove adherent ammonium*



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spontaneous fluctuations do not occur

sulfate. The precipitate is then extracted three or four times with distilled water, the insoluble material being separated each time by centrifugalization. This aqueous extract contains the active principle. The large amount of difficultly soluble material remaining is relatively inert and is discarded.

Although one-half to two-thirds saturation with ammonium sulfate effectively precipitates the active principle from crude extracts of kidneys, even complete saturation with this salt fails to remove the active principle with any degree of efficiency from more purified solutions. One is thus unable to utilize this useful procedure in further purification. The active principle is apparently only carried down as an adsorption product in the initial step of the extraction because of the presence of large amounts of contaminating proteins which are present at this stage of the process, and which are precipitated by the ammonium sulfate.

Dialyzability of the active principle. Dialysis offers a simple method for removing ammonium sulfate and other diffusible substances from solutions containing proteins or other high-molecular compounds. Several groups of investigators (2, 3) have adapted this procedure to renal extracts, injecting the solution remaining after dialysis and noting a depressor effect which they have attributed to the renal anti-pressor substance. However, the substance effective, when administered orally, in reducing the blood pressure in experimental hypertension passes through the semipermeable membrane (collodion or cellophane). The substance remaining in the membrane will reduce blood pressure of either normal or hypertensive animals but this effect is only obtained by parenteral administration. Moreover, the effect is unlike that observed following the oral administration of the substance we consider to be the physiological renal constituent. Hence, we believe the depressor action of the dialyzed material to be non-specific (6).

The active principle is recovered from the dialysate by precipitation with ten volumes of acetone or twenty volumes of absolute alcohol, or by precipitation with picric acid.

Picric acid as a precipitant. Picric acid may also be used for the elimination of water-soluble impurities (including ammonium sulfate) from the crude extract. The dried ammonium sulfate precipitate is extracted several times with a saturated solution of aqueous picric acid. The remaining solid after filtration is extracted repeatedly with normal hydrochloric acid, filtered and the filtrate precipitated with 10 volumes of acetone. The precipitate is collected, after standing in the ice-chest overnight, and washed free of picric acid.

In the presence of protein, which is present in large amounts in crude preparations of the renal extract, picric acid is an efficient precipitant of the active principle. However, it is probable that this does not involve the formation of a picrate of the active principle but merely its adsorption by the simultaneously precipitated protein. This is indicated by the failure of picric acid to precipitate the active principle quantitatively from more highly purified solutions or dialysates in which the amount of contaminating proteins are minimal. In this respect picric acid resembles ammonium sulfate as a precipitant, as already described.

The use of picric acid as just described is too laborious and entails the loss of some activity when applied to crude extracts containing large amounts of ammonium sulfate.

We have therefore preferred to apply the procedure of fractionating by partial solution in water, and precipitating with acetone prior to using picric acid for the removal of the last traces of contaminating ammonium sulfate.

Organic solvents as precipitants. The active principle is readily soluble in water and insoluble in organic solvents (ethyl alcohol, acetone, ether). Its solubility in the latter is in the order given, being much more soluble in ethyl alcohol than in ethyl ether. The solubility in aqueous solutions of organic solvents varies, depending upon the degree to which a given extract is purified. Thus, in relatively crude aqueous solutions of renal tissue, the active principle is completely precipitated by the addition of only three or four volumes of acetone or alcohol. However, in more purified concentrates, it required 10 volumes of acetone or 20 volumes of alcohol to ensure its precipitation. In this respect, the active principle behaves like many biologically important substances whose solubility in a given reagent appears to vary with the extent of their purification and freedom from contaminants.

The variation in solubility of the active principle in water and in aqueous organic solvents renders it impossible to utilize fixed concentrations in fractionating the extracts. The greater the ammonium sulfate and protein concentration of a given extract the less easily does the active principle enter solution in aqueous acetone or alcohol mixtures. On the other hand, in the case of highly purified fractions, the active principle may remain in solution in a mixture of 3 volumes of acetone and 1 of water, or in an even more concentrated alcoholic solution. Because of this variability in behavior and the lack of uniformity of different preparations it has been necessary to assay the fractions obtained by successive elutions of the dried extract or by successive precipitations with increasing amounts of organic solvent. Active fractions are then combined and the procedure repeated.

Other precipitants. We have utilized other commonly used precipitants to remove the active principle from aqueous solution—phosphotungstic acid, tannic acid, lead acetate, mercuric chloride, etc. Because of the demonstrated non specificity of such precipitants as ammonium sulfate, or picric acid, it is questionable if any of these form true combinations with the active principle It is possible that in all of these reactions the active principle is merely adsorbed on the simultaneously precipitated protein contaminants. Nevertheless, the use of these various agents has been useful in effecting a concentration of the active principle and freeing it from impurities so that in our most active preparations as little as 20 to 50 mgm. of material administered orally has lowered the blood pressure of hypertensive rats 20 to 40 mm. Hg.

DISCUSSION. One of the chief deterrents to progress in this field has been the relatively large amounts of original kidney tissue necessary to elicit demonstrable effects in lowering the blood pressure of hypertensive animals. In order to determine if greater amounts of the active principle than are extracted are actually present in the original kidney tissue, this was fed directly without preliminary extractions. In order to avoid the inevitable destructive enzymatic processes in the tissue, the fresh glands of cattle, dogs and rats, immediately on removal from the animal, were sliced and submerged in liquid air. The frozen tissue was then dehydrated *in vacuo* over phosphorus pentoxide at −30° C. The resulting white powder was admixed with the food and administered to hypertensive animals. In no case were striking drops in blood pressure observed, even when maximal amounts of the material which could be ingested were administered.

Similar results were obtained in feeding tissue obtained by dehydrating kidney tissue with a large excess (20 volumes) of acetone or alcohol, filtering the solution, washing the residue with more of the pure anhydrous solvent and drying *in vacuo* to remove the adherent solvent.

The amount of the active principle present in the kidney is apparently very small, these organs elaborating an amount sufficing for the needs of the tissues which remove it rapidly from the blood stream. In this respect the kidney resembles such endocrine organs as the adrenal cortex, ovary and testis, in which also hundreds of kilograms of tissue are required for the isolation of a few milligrams of their active constituents. The results of studies in parabiosis of rats, one of which is rendered hypertensive (8), are also in accord with this concept.

In figure 1 is reproduced a typical result obtained by feeding a concentrated fraction weighing 15 grams to a group of five rats, the pre-treatment blood pressures of which averaged 180 mm. Hg. This extract was obtained from an initial ammonium sulfate precipitate weighing 630 grams and derived from 70 kilograms of fresh pig's kidney. This precipitate was extracted with water, treated with picric acid solution and fractionated with acetone as already described. The final product was free of ammonium sulfate and was consumed by the animals without appreciable loss of body weight throughout the course of the experiment. Although the kidney from which this extract was derived was prodigious in amount, the fact that so striking a drop in blood pressure was elicited from a relatively small dose of the final product, and that this was obtained by oral administration without appreciable loss in body weight, indicates that we are dealing with a physiologically important derivative and not with any toxic (such as follows parenteral injection), starvation, or dehydration effect.

From a practical standpoint the results of the present study indicate that renal tissue contains too small amounts of the active principle to render it a practical source for therapeutic purposes. As we have previously indicated (9), it requires the product of many hundreds of pounds of kidney to lower appreciably the blood pressure of patients suffering from hypertension. Our subsequent clinical trials (10) have confirmed this conclusion. On the other hand, the behavior of the active principle, its effectiveness when administered orally, its easy passage through collodion or cellophane membranes, its solubility relations, all point to its being a compound of relatively small molecular weight. The isolation and ultimate synthesis of the material or the preparation from more readily available sources of related compounds showing a similar effect on blood pressure offer the most promising prospects for future work.

SUMMARY

Procedures are outlined for fractionating and concentrating renal extracts which, in relatively small doses administered orally, effectively reduce the blood pressure of hypertensive rats. The solubility of the active principle in water and aqueous solutions of organic solvents and the behavior of the extracts toward various precipitants (picric acid, ammonium sulfate, etc.) are described. The dialyzability of the active principle differentiates it from the agent described by other workers as effective following parenteral administration.

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THE POTENCY OF A NEW EXTRACT OF CONVALLARIA MAJALIS LEAVES

III. Assays by the Papillary Muscle Procedure and the U.S.P. XII Cat Method

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During the past few years there has been a renewed interest in therapy with standardized extracts of *Convallaria majalis* leaves, at first in Europe (1, 2, 3, 4), and then in the United States (5, 6). Our preparation[2] was made by a method similar to that described by Straub (7). It consisted of an aqueous extract of the leaves, treated with ferric hydroxide to remove tannins and gums, and then concentrated under reduced pressure until 1 gram of the extract was equivalent to 30 grams of dried leaves. In our first publication on this extract (8), we determined its potency by the 18-hour frog method and found it stable, even in aqueous solution at room temperature. We then tested it using the pigeon emesis method (9).

It was shown by Cattell and Gold (10) that certain cardiac glucosides increased the strength of the isometric contractions of the isolated papillary muscle of the cat heart. We compared this convallaria extract (Convara) with the U.S.P. XI reference digitalis by their method. Furthermore, we assayed the extract by the U.S.P. XII Cat Method, comparing it with both the U.S.P. XI and the U.S.P. XII reference standard digitalis.

EXPERIMENTAL PROCEDURES: *1. Papillary muscle.* Our technique was similar in principle to that introduced by Cattell and Gold (10), employing a somewhat simpler mechanical arrangement. Although not as adaptable as that used by the above authors, our arrangement has proved very satisfactory for comparison of the strength of drugs by this method. The muscle was mounted in an ordinary muscle warming chamber; oxygen was bubbled in at the bottom of the liquid from an inflated toy balloon. Twenty-five cc. of a modified Locke's solution, as described by Cattell and Gold (10), adjusted to pH 7.2 were put into the chamber. The warming chamber was immersed in an automatic Sargent constant temperature bath, maintained at 37°C. throughout the experiment. When the papillary muscle was dissected from the heart, ventricular muscle and heart-valve tissues were left attached. The muscle was then tied by thread, the attachments being made to these tissues, the valve-end passing to the isometric lever. This lever was attached to a spring-metal band (tension adjustable), upon which a small plane mirror was mounted. Contraction of the muscle caused a slight movement of the mirror The movements of the mirror were recorded by photographing the reflections of a strong beam of light on electrocardiograph film mounted on a drum of a kymograph, the kymograph was enclosed in a box with a slit about one millimeter in width in alignment with the beam of light The muscle was stimulated by a condenser discharge at a

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[1] George A. Breon Fellow in Pharmacology, 1941–1942.

[2] This preparation, Convara, was furnished by Dr. C. W. Sondern of George A. Breon and Company, Kansas City, Mo.

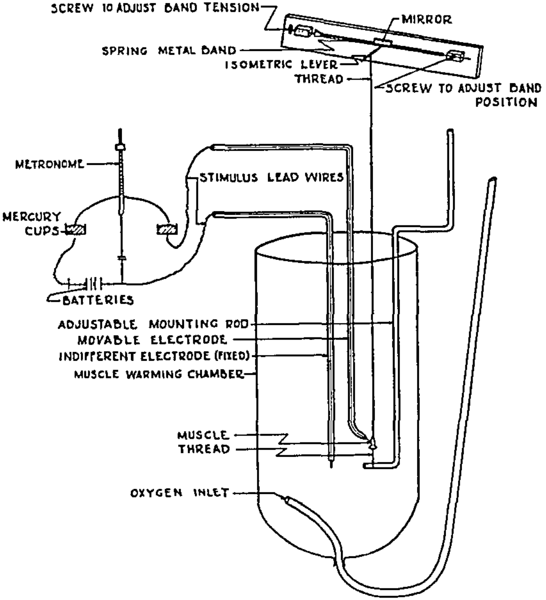
SCREW TO ADJUST BAND TENSION
MIRROR
SPRING METAL BAND
ISOMETRIC LEVER
THREAD
SCREW TO ADJUST BAND POSITION
METRONOME
STIMULUS LEAD WIRES
MERCURY CUPS
BATTERIES
ADJUSTABLE MOUNTING ROD
MOVABLE ELECTRODE
INDIFFERENT ELECTRODE (FIXED)
MUSCLE WARMING CHAMBER
MUSCLE
THREAD
OXYGEN INLET

FIG 1 SCHEMATIC VIEW OF THE MAIN PARTS OF THE APPARATUS USED IN THE STUDIES UPON PAPILLARY MUSCLE

rate of 40 times per minute Since the papillary muscle follows the all or none response a stimulus of sufficient strength to produce regular responses was employed Stimuli varying in strength to produce regular responses was employed Stimuli varying in strength from 0 25 to 1 0 (usually 0 75) microfarads generated with a 45 v 'B' battery, were used In most of the experiments the stimuli were led in through a wire within a small bore glass tubing with nichrome tips sealed in at the end One acted as an 'indifferent electrode" inserted in the

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TABLE 2

Assays of convallaria extract (Convara, 1:1800) and of U S P. XI (1936) reference tincture of digitalis (6%) by the U.S.P. XII intravenous cat method

U.S.P. XI REF. TINCT OF DIGITALIS		CONVARA	
Cat no.	Death in no. of injections	Cat no	Death in no. of injections
24	16	21	14
26	13	25	16
29	11	27	10
30	12	28	12
32	14	31	14
34	16	33	11
36	14	35	14
37	12	38	17
40	11	39	16
42	14	43	16
Averages...... ...	13 3 ±0.58 (4.4% of average)		14.0 ±0.75 (5.4% of average) 7.8 mgm./kgm.

Equivalence of 0.1 gram of Convara: 8 6 ±0 59 U S.P. XI (1936) digitalis units.

The digitalis group consisted of 9 males and 1 female, average weight, 2.93 kgm., the convara group of 8 males and 2 females, average weight, 2.75 kgm.

The cats were kept a minimum of 10 days in our laboratory before the assays and all tests were carried out between May 26th and June 4th, 1942.

TABLE 3

Assays of convallaria extract (Convara, 1:1900) and of U.S.P. XII (1942) reference tincture of digitalis (6.6%) by the U.S.P. XII intravenous cat method

U.S.P. XII REF. TINCT. OF DIGITALIS		CONVARA	
Cat no	Death in no. of injections	Cat no	Death in no of injections
46	13	45	14
47	17	48	13
49	14	51	12
50	18	53	13
52	15	54	15
55	14	56	12
59	14	58	13
61	17	60	16
63	16	62	15
65	13	64	13
Averages .	15.1 ±0 57 (3 8% of average)		13.6 ±0.43 (3.2% of average) 7.2 mgm./kgm.

Equivalence of 0 1 gram of Convara · 11 8 ±0 6 U.S P. XII (1942) digitalis units.

The two groups each had 5 male and 5 female cats; the average weights were 2 60 and 2 50 kgm., respectively. The cats were kept a minimum of 2 days in our laboratory before the assays and 19 of the 20 assays were completed within a week of arrival of the animals. All tests were carried out between June 8 and 13, 1942, or within 6 days.

were taken every five minutes throughout the experiment until the death of the muscle.

Cattell and Gold, in a later publication (11), extended their work on the papillary muscle. In this second paper the relation of "therapeutic" to "toxic" concentrations of several cardiac glucosides were studied. The "therapeutic" concentration was arbitrarily chosen as that concentration of the drug which produced an increase in response in approximately fifty per cent of the experiments. Higher, "toxic" concentrations produced a comparatively rapid increase in the force of contraction followed by a fall and ultimate failure of the muscle, often accompanied by spontaneous contractions or missed responses. Although some of the glucosides studied differed among themselves in potency, nevertheless, the ratio between the toxic concentration and the therapeutic concentration seemed to be approximately the same. In view of this observation, and because values for the "therapeutic" concentrations are difficult to determine accurately, our experiments were conducted in the "toxic" range only. From a series of preliminary trials, concentrations of Convara and U.S.P. XI reference standard tincture of digitalis were selected to give approximately the same activity. The final ten experiments then were conducted with each drug. In accord with the recommendation of Cattell and Gold (11), the criterion for measuring the toxic effect was the time elapsing between the addition of the drug and restoration of the muscle response to a systolic tension of one-half the maximum. Our data are presented in table 1.

2. Assays by the cat method. Finally, we also assayed the Convara by the cat method according to the procedure given in the U.S.P. XII for the assay of tincture of digitalis (12). Two complete assays were conducted, one using U.S.P. XI (1936), and the other, U.S.P. XII (1942), reference digitalis; the former enabled us to compare the results of the cat method with those of the frog, pigeon, and papillary muscle, respectively, and the latter gave an evaluation in terms of the present official standard.[3]

Briefly, the procedure consisted in anesthetizing each cat with ether, inserting a cannula into a femoral vein, and injecting 1 cc. of suitably diluted drug per kgm. of body weight once every 5 minutes under light ether anesthesia. The reference tinctures of digitalis, and Convara were diluted with 0.9% solution of sodium chloride so that the average number of injections required to produce cardiac arrest in each assay group would fall between 13 and 19. The data are presented in tables 2 and 3.

Discussion. The results secured by the two methods presented in this article, as well as those previously obtained by the 18-hour frog and pigeon emesis methods (8, 9), were conducted in the same laboratory by the same operators, using material from the same sample of convallaria extract. Therefore, the data on the different assay methods should be reliable for comparative purposes. The potencies of 0.1 gram of the convallaria extract expressed in U.S.P. XI digitalis units for the four methods of assay are given in table 4.

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[3] In all cases the tincture of the U.S.P. XI Reference Digitalis Powder was made to contain 0.745 gram per 10 cc. and that of the U.S.P. XII, 1.000 gram per 10 cc.

Of the 4 methods, the 18 hour frog method gives the highest value Then follow the intravenous cat method, and finally the pigeon emesis and the papillary muscle methods, the latter two being about equal in value Except for the pigeon emesis and papillary muscle technique, the differences among the results of the assays are significant It has been stated (13) that, because of the more rapid elimination or destruction of convallaria glucosides, or both, as compared to digitalis glucosides in warm blooded animals, the U S P XII cat method will give much smaller values than the frog method Our experiments do show that the cat method gives a lower value than the frog assay, but this difference was not as great as expected However, when longer infusion times are employed, it has been demonstrated that convallaria preparations are much more rapidly detoxified than those of *Digitalis purpurea* (14, 15, 16) Accordingly it is possible that, if slower infusion rates were employed with the cat, or greater dilutions of the drugs were used, the potency of the convallaria extract would be still less in relation to the frog values

TABLE 4

Potency of convallaria extract (Convara) by different assay methods

METHOD	POTENCY (EQUIVALENCE OF 0 1 GRAM CONVARA IN U S.P XI (1936) DIGITALIS UNITS)
Eighteen hour frog	10 1 ±0 5
Pigeon emesis	6 1 ±0 37
Intravenous cat	8 6 ±0 59
Papillary muscle	6 1 ±0 8

Furthermore, there is another factor, the relative amounts of the saponins present, which may influence the potencies of a plant extract as shown by different methods of assay, since it was shown (17) that addition of saponin (in non toxic concentration) to the infusion fluid acted to raise the toxicity of strophanthin 28 7% Saponins are present in both digitalis and convallaria It is not known how these substances may influence the other methods of assay

One other fact that this study has brought out is the difference between the U S P XI (1936) and XII (1942) digitalis units when compared by the intravenous cat method Our experiments show that the potency of the U S P XII digitalis unit is only 78 6 ± 4 5% of the U S P XI digitalis unit However, this value is only approximate because the cats were not kept the same length of time in our laboratories and we do not know how long they had been kept in the quarters of the animal dealer in Wisconsin who supplied them to us Considering this and the rather small number of cats in each series, our results agree quite well with those of Miller, who reported a difference of 16% (18)

SUMMARY

1 A simplified modification of the apparatus devised by Gold and Cattell for testing the potency of cardiac drugs by their action on the isolated papillary muscle of the cat is presented

2. The potency of a concentrated, purified, aqueous extract of the leaves of Convallaria majalis (Convara) was found equivalent to 6.1 ± 0.8 U.S.P. XI digitalis units per 0.1 gram by its activity on the isolated papillary muscle.

3. The potency of the same extract was found equivalent to 8.6 ± 0.6 U.S.P. XI (1936) digitalis units and 11.8 ± 0.6 U.S.P. XII (1942) digitalis units, respectively, per 0.1 gram by the U.S.P. cat method.

4. The U.S.P XII digitalis unit was about 79% the strength of the U.S.P. XI digitalis unit when tested by the cat method.

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THE OVERT AND MASKED MANIFESTATIONS OF FOLLICULOID HORMONES

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The most commonly used indicator of the so-called "estrogenic" or folliculoid hormones is their ability to induce cornification of the vaginal epithelium in spayed rodents. Since under certain conditions (vitamin A deficiency or mechanical irritation of the vagina etc.) vaginal cornification may be produced without inducing any other signs of estrus, it appears inadvisable to gauge the activity of the follicular hormones by this single manifestation. The vagina-cornifying effect is a particularly poor index of folliculoid activity of compounds which are also endowed with other hormonal properties since it is known that luteoids, corticoids and testoids induce vaginal mucification and inhibit the cornifying effect of the folliculoids. Following administration of these hormones there is a transitory period of vaginal cornification and stratification followed by continuous mucification. Using this transitory cornification as a test object in a series of 49 steroids—selected more or less at random—it has been demonstrated that all hormonally active compounds (folliculoids, luteoids, corticoids, testoids) exert some degree of folliculoid activity irrespective of their main hormonal actions. Indeed it has been emphasized that the folliculoid activity—just as the anesthetic activity—may be a property common to all steroid hormones (1). The ability to prevent the formation of castration cells in the pituitary of gonadectomized male or female rodents (2), to stimulate the growth of the uterus and mammary glands (1) and to induce atrophy of the Leydig cells in the testis (3) have likewise been shown to be properties common to the entire steroid hormone group. The anesthetic effect of any one compound is in no way proportional to the other actions mentioned above, but there appears to be a certain parallelism between the ability of compounds to prevent the formation of castration cells and to cause vaginal cornification, uterine enlargement, mammary gland stimulation and Leydig cell atrophy. Since only the folliculoid estratrienes elicit these actions to the exclusion of all other hormonal effects and since they are so very much more active than any other hormones, these effects have been considered characteristic of and directly subordinate to the folliculoid potency.

In order to obtain further data in support of this hypothesis we examined a number of steroids belonging to different hormonal groups and some devoid of all known hormonal activities. We studied their effect in spayed immature rats on vaginal opening and cornification, mammary gland stimulation, uterine weight and castration changes in the pituitary. If all these effects are really subordinate to each other and represent merely different manifestations of folliculoid activity, they should run parallel with each other; that is to say, a compound very active with respect to one of these actions should also be very active with respect to any of the other properties enumerated above. Furthermore, all compounds exhibiting any type of hormonal activity should possess some degree of folliculoid potency, while all hormonally inactive compounds should be devoid of it.

Experimental. All our experiments were performed on immature, female, albino rats weighing 40–60 grams (average 50 grams) at the onset of the experiment. They received the compound to be tested in 0.1 cc. of peanut oil subcutaneously twice daily during the period of 10 days, injections being started 24 hours after ovariectomy. On the 11th day they were sacrificed and their organs fixed in "Susa" solution for weighing and subsequent histological study. All organs were weighed after fixation partly because this helps to preserve them in good condition for microscopical examination, and partly because weights of organs weighed wet at autopsy are subject to a variable loss of water during the process. With our technic it was possible to fix all tissues immediately after death and to keep them moistened by the fixative during dissection, drying them only one organ at a time for the short period necessary for the actual weighing.

The details of our data are presented in table 1 in which the steroids are described by their popular names (in block letters); their systematic chemical names as well as the melting point of the sample employed by us are also mentioned to facilitate the identification of the compounds[1] used and to give a rough estimate of the degree of purity of our material. The total daily dose administered during the 10-day injection period and the average final body weight (with the range in brackets) are given in the next two columns. The rest of the table is concerned with the various hormonal effects observed. On each dose level of the 31 groups 6 rats were used and the data recorded in the table indicate the average degree of activity observed within the group. We noted the number of rats in which the vagina opened during the 10-day injection period, the number of days of treatment which elapsed before the vagina opened and the character of the vaginal smears during the experiment as well as its histological appearance after autopsy. The vaginal smears could only be taken, of course, in animals in which the vaginal membrane ruptured during treatment. The fully cornified smear was marked + while other smears were indicated by a minus sign. It is essential to differentiate between compounds causing continuous cornification and those eliciting only transitory vaginal estrus. Hence the injection period was subdivided into an initial stage (first 2 days after vaginal opening) and final stage (remainder of the experiment) each of which was separately marked by the above symbols, that is – – means continuous diestrus, +– transitory estrus and ++ continuous estrus, no markings being given for periods during which the vagina was closed. The histological structure of the vaginal epithelium was recorded by the following abbreviations. A = anestrus, S_T = trace of stratification, S_1 = slight stratification, S_2 = moderate stratification, S_3 = marked stratification. C was used to indicate cornification and M for mucification, the degrees of these transformations being indicated in the same manner as in the case of stratification. The changes in the mammary glands are considered under the headings of secretion, duct growth and acinar growth, the degree of each of these changes being indicated by an arbitrary scale of 0–4 in which *0* is the condition in a spayed female and *4* the maximum possible change. It will be noted that mammary gland changes develop comparatively slowly under the influence of hormones and require fairly high concentrations, hence full development was not observed in this experimental series with any of the hormones. The average weight of the preputial glands and uteri in each group are given with their standard errors (in brackets), the weights which are significantly above those of the control group (injected with peanut oil only) are given in larger print Since in the case of small organs minor differences in the method of dissection, length of fixation, water evaporation during weighing etc are very likely to induce significant variations, it was deemed advisable not to consider any deviation as significant unless *P* equalled 0 01 or less, that is to say unless the observed deviation from the normal could occur by chance no more than once in 100 trials It should be emphasized that the preputial glands are not stimulated by folliculoid compounds but represent a special manifestation of the testoid activity (4, 5) Their weight is recorded here merely for comparative purposes since like the mammary glands the preputial glands are modified skin glands and hence are somewhat related to the former It is quite obvious from the table, however, that their responsiveness to hormones is entirely different In the last column of the table the ability of the compounds to prevent the formation of castration cells is registered by means of a scale of 0–3, *0* meaning that the castration changes were just as marked as in the pituitaries of the peanut oil injected controls, while *3* indicates that they were completely prevented

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[1] In accordance with International Union Rules for the Naming of Organic Compounds, positions in the side chain are numbered from the point of attachment by numerals with indices (*i.e.* 17^1, 17^2, etc.); for the sake of brevity, however, side chain substituents are enumerated with nuclear substituents rather than bracketed with the substituted alkyl radical. This represents a slight deviation from the previously used (1) system.

It will be noted that the control group is mentioned first, the other groups being arranged according to increasing mean uterine weights irrespective of the compound or dosage used Thus the particular dosages at which the various hormones exert a comparable degree of uterine stimulation occupy a neighboring position in our table The uterus enlarging effect of folliculoids, like the vagina cornifying effect, can be "masked" by the other hormonal effects of a compound (1) This introduces complications because in certain cases such inhibitable actions are less evident at high than at low dose levels It has been found in several instances that in the case of hormone mixtures or of compounds having several hormonal actions the folliculoid effects are readily demonstrable at low dose ranges, while comparatively high doses are required for the inhibitory or ' anti folliculoid" effect (1, 6, 7) In spite of this complicating factor the uterine weight was chosen as an indicator of folliculoid activity because it lends itself to statistical analysis and hence represents an objective indicator

Perusal of table 1 clearly shows that when the groups are arranged without any consideration of their chemical structure or the dosage given, but according to increasing degrees of uterus stimulating activity, they automatically also arrange themselves according to increasing vaginal, mammary gland stimulating and anti castration cell potencies In other words in the compounds tested these three activities run roughly parallel with each other Thus it may be said, for example, that 1 mgm of methyl testosterone (group 24) is approximately as effective as 2 mgm of ethinyl testosterone or 0 1 γ of ethinyl-estradiol with regard to all folliculoid activities, while no such parallelism exists between the effect of these three compounds on the preputial glands The same considerations hold true concerning the effect of 1 γ of estrone (group 28) in comparison with 2 mgm of androstenediol or 1 mgm of testosterone In this manner the folliculoid activity of various hormones can be expressed roughly in terms of ethinyl estradiol or estrone equivalents Obviously the parallelism between the various manifestations of folliculoid activity is not absolute, partly because the uterine activity is not always overt and partly because, especially at high dose levels, the accuracy of the assay method is poor Thus it will be noted that there is no significant difference between the action of estradiol (group 26) at the dose ranges of 0 5 to 10 γ while 0 1 γ (group 15) is very much less effective With regard to the self inhibition of the uterus enlarging action

Morphological changes induced by various steroids in spayed rats

NO. OF COM-POUND	STEROID	M.P. (°C)	DOSE/DAY	AVERAGE FINAL BODY WEIGHT (G.)	VAGINAL OPENING		VAGINAL SMEARS		VAGINAL HISTOLOGY	MAMMARY GLAND			PREPUTIAL GL. WEIGHTS (MG.)	UTERINE WEIGHTS (MG.)	PITUITARY ANTI-CASTRATION CELL EFFECT
					No. open in 10 days treatment	No. of days of treatment before vagina opened	1	2		Secretion	Duct growth	Acinar growth			
	PEANUT OIL		0.2 cc.	90 (84-94)	0				A	0	0	0	21 (1.5)	27 (3.7)	0
1	*ANDROSTERONE* Androstane-*3*(α)-ol-*17*-one	177-178	0.1 mg.	74 (62-80)	2	*10*	—		A	*0*	0	*0*	*34* (3.2)	*24* (2.5)	0
2	*CIS-TESTOSTERONE* Δ^4-androstene-*3*-one-*17*(β)-ol	215-217	2 mg.	78 (61-90)	2	5	—	—	S_1	0	0	0	*45* (8.3)	25 (1.3)	0-1
3	*ETIOCHOLANOLONE ACETATE* Etiocholane-*3*(β)-ol-*17*-one acetate	157	10 mg.	74 (71-78)	0				A	0	0	*0*	21 (2.5)	*27* (3.7)	0
4	Δ^5-*PREGNENOLONE* *17*-ethyl-Δ^5 androstene-*3*(β)-ol-17^1-one	188-189	2 mg.	83 (74-88)	0				A	0	0	*0*	31 (4.6)	*28* (1.6)	0
5	*ACETOXYPREGNENOLONE* *17*-ethyl-Δ^5 androstene-*3*(β), 17^2-diol-17^1-one-17^2-acetate	182 183	2 mg.	90 (80-100)	0				A	0	0	0	*27* (1.3)	28 (2.1)	0
6	Δ^{16}-*PREGNENEDIONE* *17*-ethyl-Δ^{16}-etiocholene-*3*, 17^1-dione	197 198	2 mg.	93 (82-112)	0				A	0	0	0	*27* (2.4)	*29* (0.5)	0
7	*PREGNANEDIOL* *17*-ethyl-etiocholane-*3*(α), 17^1(α)-diol	237	2 mg.	86 (70-96)	1	9	—	—	A	0	0	0	25 (2.2)	30 (3.4)	0
8	$\Delta^{5,16}$-*PREGNADIENOLONE ACETATE* *17*-ethyl-$\Delta^{5,16}$-androstadiene-*3*(β)-ol-17^1-one acetate	173-174	2 mg.	93 (83-105)	1	7	—	—	A	0	0	0	*35* (2.5)	31 (2.4)	0
9	*ANDROSTANE*	48.5-50	2 mg.	89 (79-97)	0				A	0	0	0	23 (2.2)	32 (2.4)	0

10	*ANDROSTENEDIOL-3(β) 17(β)* Δ^5-androstene-3(β) 17(β)-diol	203 204	10 mg	80 (60-91)	1	5	—	—	A	0	0	0	*80* (8 8)	32 (3 2)	0-1
11	*ESTRONE* $\Delta^{1\,3\,5}$-estratriene-3-ol 17 one	252 254	0 1 γ	85 (74-95)	0								23 (1 3)	33 (1 1)	
12	*ANDROSTERONE* Androstane-3(α)-ol 17-one	179-180	1 mg	88 (83-92)	3	7	—	—	A	1	1	0	*58* (1 7)	34 (5 5)	1
13	*ACETOXYPREGNENOLONE* 17-ethyl Δ^5-androstene-3(β) 17^2-diol 17^1-one-17^2-acetate	177 178	10 mg	90 (80 95)	1	10	+		A	0	0	0	23 (2 0)	38 (1 5)	0
14	*DESOXYCORTICOSTERONE ACETATE* 17-ethyl Δ^4 androstene-3 17^1 dione 17^2-ol 17^2-acetate	152	2 mg	92 (80 99)	0				A	0	1	0	26 (1 3)	39 (3 0)	0
15	*ESTRADIOL* $\Delta^{1\,3,5}$ estratriene-3 17(α) diol	176	0 1 γ	83 (75-95)	5	10	+		$\mathrm{S\,M_T}$	0	0	0	26 (1 9)	*44* (2 8)	0 1
16	*ANDROSTENEDIOL-3(β) 17(β)* Δ^5 androstene 3(β) 17(β) diol	203 204	2 mg	87 (75 94)	6	2	•	—	A	0	1	0	*51* (2 6)	*46* (1 8)	0
17	*Δ^5 PREGNENOLONE* 17 ethyl Δ^5 androstene 3(β)-ol 17^1 one	185 186	10 mg	80 (68 93)	1	10	—		$\mathrm{S_T}$	0	0	0	*48* (5 8)	*45* (4 6)	0
18	*PROGESTERONE* 17 ethyl Δ^4 androstene 3 17^1 dione	128	10 mg	73 (70 75)	2	8	—	—	$\mathrm{S_TM_T}$	0	0	0	*48* (8 6)	*47* (3 9)	1 2
19	*DESOXYCORTICOSTERONE ACETATE* 17 ethyl Δ^4 androstene-3 17^1 dione 17^2-ol 17^2-acetate	152	10 mg	80 (67 100)	0				A	0	0	0	30 (3 2)	*49* (5 4)	0
20	*PROGESTERONE* 17-ethyl Δ^4-androstene-3 17^1-dione	128	1 mg	84 (74 93)	0				$\mathrm{S_TM_T}$	0	1	0	44 (9 6)	*58* (8 8)	0
			2 mg	79 (69-84)	2	8	•	•	A	0	1	0	*42* (3 1)	*56* (2 5)	1
21	*ANDROSTENEDIONE* Δ^4 androstene-3 17-dione	169	2 mg	*82* (72 100)	5	9	+	—	$\mathrm{S_1}$	*0*	1	*1*	*64* (8 3)	*87* (4 0)	*0-1*

• Vaginal smears were not observed at this time

TABLE 1—*Concluded*

NO OF COM-POUND	STEROID	M P. (°C)	DOSE/DAY	AVERAGE FINAL BODY WEIGHT (G)	VAGINAL OPENING		VAGINAL SMEARS		VAGINAL HISTOLOGY	MAMMARY GLAND			PREPUTIAL GL. WEIGHTS (MG.)	UTERINE WEIGHTS (MG.)	PITUITARY ANTI-CASTRATION CELL EFFECT,
					No. open in 10 days treatment	No. of days of treatment before vagina opened	1	2		Secretion	Duct growth	Acinar growth			
22	*DEHYDRO ISO-ANDROSTERONE* Δ^5 androstene-*3*(β) ol-*17*¹ one	140	2 mg	88 (80 96)	6	3	•	•	S_2M_1	0	1	1	*78* (5 0)	*69* (3.8)	2
,23	*ETHINYL TESTOSTERONE* *17* ethinyl Δ^4 androstene-*3*-one *17* ol	265 268	2 mg	88 (73 95)	6	2	+	+	S_2C_1	0	1	1	*48* (2.8)	*118* (3.9)	2
24	*METHYL-TESTOSTERONE* *17* methyl Δ^4 androstene-*3*-one-*17*-ol	153	1 mg.	77 (70-84)	6	4	+	−	S_2M_2	0	2	1	*71* (5.8)	*138* (8 3)	2
25	*ETHINYL ESTRADIOL* *17*-ethyl $\Delta^{1\,3\,5}$ estratriene *17*¹ ine-*3*,*17*-diol	145-146	0.1 γ	70 (67-75)	6	5	+	+	S_2C_1	0	0	0	25 (*1.8*)	*145* (*11*)	2
26	*ESTRADIOL* $\Delta^{1\,3\,5}$ estratriene *3*,*17*(α) diol	176	2 γ	81 (78 83)	6	4	+	+	S_2C_2	2	1	2	26 (1.5)	*146* (10)	2-3
			0 5 γ	78 (67-85)	6	5	+	+	S_2C_2	1	1	1	23 (1.9)	*160* (8.0)	2
			5 γ	74 (60 82)	6	3	+	+	S_2C_2	2	1	2	25 (3.2)	*158* (8.3)	2-3
			10 γ	80 (67-92)	6	3	+	+	S_2C_2	2	1	2	*31* (1.8)	*158* (8.3)	3
27	*ANDROSTENEDIOL* Δ^5 androstene *3*(β),*17*(α) diol	180-181	2 mg	80 (71-89)	6	3	+	+	S_2C_2	0	1	0	*88* (7.1)	*165* (7.5)	2 3
28	*ESTRONE* $\Delta^{1\,3\,5}$ estratriene-*3*-ol-*17* one	252 254	1 γ	77 (71-80)	6	5	+	+	S_1C_2	0	0	0	25 (1.9)	*169* (19)	3

29	*TESTOSTERONE* Δ^4 androstene-*3*-one-*17*(α)-ol	154	1 mg	88 (79–94)	6	5	•	–	S_1M_1	2	2	1	*67* (3 9)	*178* (19)	2
			2 mg	90 (74–118)	6	5	+	–	S_1M_2	1	2	1	*92* (9 1)	*187* (14)	2
30	*ETHINYL ESTRADIOL* *17*-ethyl $\Delta^{1,3,5}$-estratriene *17*-ine-*3* *17* diol	176	5 γ	75 (60-79)	6	4	+	+	S_2C_2	2	2	2	28 (2 8)	*190* (13)	3
			0 5 γ	73 (66–82)	6	5	+	+	S_3C_3	0	0	0	21 (0 9)	*192* (8 6)	3
31	*TESTOSTERONE PROPIONATE* Δ^4 androstene-*3*-one *17*(α)-ol	119	1 mg	83 (71 102)	6	5	+	–	S_1M_2	2	2	2	*104* (7 5)	*226* (6 3)	3

of some compounds at high dose levels, it should be pointed out that androstenediol-3(β),17(β) at a 2 mgm. dose level (group 16) is more effective than at a 10 mgm. dose level (group 10) and progesterone is more active at the 1 or 2 mgm. dose level (group 20) than at the 10 mgm. dose level (group 18). Conversely certain folliculoid activities (*e.g.* the mammary gland stimulating effect) may be augmented by other hormonal actions of the molecule.

In order to avoid these complications and to give an overall estimate of the folliculoid activity of various compounds, a summary table (table 2) was constructed in which the mean rating for each of the four folliculoid effects (vaginal, uterine, mammary and pituitary) was calculated in the following manner. For an estimate of total vaginal stimulating activity an arbitrary scale of *0–4* was employed; *0* meaning that no effect was observed; *1*, that the vagina of some of the animals opened but on histological examination the epithelium was atrophic; *2*, that the vagina of some animals opened and the epithelium was somewhat stratified; *3*, that the vagina of all animals opened but that its epithelium did not remain continuously cornified; *4*, that the vagina of all animals opened and the epithelium remained cornified continuously. For comparing uterine activities the mean weights were used. To obtain an overall estimate of mammary stimulating activity another arbitrary scale of *0–4* was employed; *0* indicating that the compound showed no activity at a given dose level; *1*, slight acinar and/or duct stimulation; *2*, more marked acinar and duct stimulation but no secretion; *3*, moderate growth with secretion; *4*, marked growth with considerable secretion. The ability of the compounds to prevent the formation of castration cells was registered by means of the same scale as was employed in table 1.

The compounds were arranged according to decreasing "overall folliculoid activity" their position in the series being determined on the basis of comparisons between the minimum effective threshold levels obtained by the above-mentioned purely mathematical computation of the four activities at the lowest active dose range. The steroids were then placed in decreasing order of each type of activity. Five columns were thus constructed enumerating the compounds according to decreasing anticastration cell, uterus stimulating, vaginal, mammary gland stimulating and "overall" folliculoid activity. Whenever two or more compounds happened to exhibit the same degree of activity they were given the same serial number. The numerals corresponding to the positions in the series which thus became free were not used. In the sixth column the preputial gland stimulating activities were similarly indicated.

Inspection of the table shows clearly that a surprisingly close correlation exists between the four individual subordinate folliculoid activities and the overall estimate. Three of the non-hormonal steroids are not discussed in table 1 since they were assayed on rats slightly different in body weight from the remaining animals of the series, but they are included in table 2 since they all had their own controls. In agreement with expectations all hormonally inactive steroids were devoid of folliculoid effects, while all hormonally active compounds exhibited these in varying degrees irrespective of the nature of their hormonal actions. It will be recalled, however, that in agreement with the natural classification of the steroids which was recently proposed (8) folliculoid activity decreases among the compounds possessing predominantly folliculoid (*e g* estradiol), testoid (*e g* testosterone), luteoid (*e g* progesterone), corticoid (*e g* desoxycorticosterone acetate) and anesthetic (*e g* pregnanedione) properties in this order

Acetoxy pregnenolone and desoxycorticosterone acetate assayed somewhat lower in folliculoid potency than in some of our earlier experiments (2) which may be due to differences in the purity of the batches or in the experimental technic employed in the two investigations, but since both in earlier and in the present series the compounds proved to be the least folliculoid of all hormonally active steroids these minor differences are not of fundamental importance It may also be stated that although at the dose levels indicated in table 1 desoxy corticosterone acetate exhibited no anti castration cell effect, an additional experiment on two spayed females in which 40 mgm of the compound were given over a period of 10 days definitely confirmed that if sufficient quantities are given the compound possesses this activity

TABLE 2

Relative hormonal activities of different steroids

NO OF CPD	STEROID	PITUITARY ANTI CASTRATION CELL ACTIVITY	UTERUS STIMULATING ACTIVITY	VAGINAL ACTIVITY	MAMMARY GL STIMULATING ACTIVITY	OVERALL ESTIMATE OF FOLLICULOID ACTIVITY	PREPUTIAL GLAND STIMULATING ACTIVITY
1	Ethinyl estradiol	1	1	1	2	1	
2	Estrone	2	2	1	3	2	
3	α Estradiol	3	2	3	1	3	
4	Testosterone propionate	4	4	6	4	4	1
5	Testosterone	6	5	6	5	4	2
6	Androstene-*3*(β),*17*(α) diol	5	5	4	9	6	4
7	Methyl testosterone	6	7	6	7	7	2
8	Ethinyl testosterone	6	8	4	9	8	9
9	Dehydro *iso* androsterone	6	12	6	9	9	4
10	Androsterone	10	9	10	6	10	7
11	Progesterone	11	10	13	8	11	9
12	Androstenedione	12	10	11	9	11	6
13	Androstene-*3*(β),*17*(β) diol	14	13	15	9	13	8
14	Cis testosterone	12	16	11	15	14	9
15	Desoxycorticosterone acetate	15	13	17	9	14	
16	Δ^5 Pregnenolone	16	15	14	15	15	
17	Acetoxypregnenolone			16			
18	Pregnanediol			16			
19	Androstane						
20	Pregnanedione						
21	Δ^{16} Pregnenedione						
22	$\Delta^{5\ 16}$ Pregnadienolone						
23	Etiocholanolone acetate						
24	Cholesterol						
25	Δ^4 *Nor* cholestene *3 25* dione						

SUMMARY

*Experiments on immature, spayed, albino rats indicate that in a group of 25 steroids the ability to produce vaginal estrus, stimulation of the mammary gland, enlargement of the uterus and to prevent the appearance of castration cells in the pituitary runs roughly parallel. In other words compounds inactive in one of these respects are also inactive with regard to the other actions mentioned and if the compounds are arranged according to increasing order of activity for one of these actions they automatically are also arranged approximately in increasing order of activity for the other actions. This supports the theory according to which the above effects are merely different manifestations of the same type of action.

Generally speaking all these effects are by far most pronounced in the predominantly folliculoid or "estrogenic" type of hormones and less pronounced in the predominantly testoid, luteoid, corticoid and anesthetic steroids, the activity diminishing in the order mentioned. This is in agreement with their expected activities as predicted by their position in the systematic table of the steroids (8).

All hormonally active compounds, but none of the hormonally inactive steroids tested, were found to possess some degree of folliculoid potency. This supports the view that the folliculoid activity is a common fundamental property of all hormonal steroids.

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DOSAGE RELATIONSHIPS IN AUGMENTATION OF PITUITARY GONADOTROPIC EXTRACT BY BLOOD AND HEMIN[1]

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Augmentation of the action of anterior pituitary gonadotropic extracts has been obtained with serum (1), copper and zinc salts (2), zinc salts (3), formed element fraction of blood (4), and hemin (5) The criterion for augmentation has been the greater weight of the ovaries of immature rats which have been injected with the augmenter plus the pituitary gonadotropic extract as compared with those of rats injected with only the gonadotropic extract

Reported studies of augmentation have been concerned with the determination of substances having augmenting powers and not with such factors as might influence augmentation quantitatively It is the purpose of this paper to report data concerned with the relationship of quantity of augmenter and quantity of pituitary gonadotropic extract to augmentation of ovarian weight and decrease of percentage ovarian solids in immature female rats

MATERIALS AND METHODS Two unfractionated gonadotropic extracts of whole sheep pituitary glands were used in this study One was made by the method described by Fevold, *et al*, (6) which involves the use of 50% aqueous pyridine The 33% acetone soluble fraction of the aqueous pyridine extract was used The other was prepared by extraction of acetone desiccated whole gland with water at pH 7 6 Supercentrifugation of this extract resulted in the removal of considerable inert material

The augmenters used were cow blood and hemin prepared from cow erythrocytes according to the method of Drabkin and Austin (7) The blood pituitary mixtures were prepared *for injection by the addition of the blood to the pituitary extracts* *The dosages of* pituitary extracts were based on the milligram equivalents of whole dry pituitary gland powder

Since hemin is not soluble in a neutral aqueous medium it was necessary to add 0 5% sodium carbonate to insure its solubility The same quantity of alkali (Na_2CO_3) was used in the preparation of the pituitary dosages which were not mixed with hemin

Five different dosages of the 33% acetone soluble anterior pituitary extract (25 50 100, 200 400 mgm equiv) were each combined with six different dosages of cow blood (0 0 0 2 0 4 0 8 1 6 3 2 cc) Thus, a total of thirty different mixtures of blood and pituitary extract were used Each of these mixtures was administered subcutaneously to three 21 day old rats 0 5 cc being injected twice daily for four and one half days and the rats killed 16 to 18 hours after the last injection

For the purpose of analyzing the data, the rats which received the different dosages of anterior pituitary extract without blood (0 0 cc) were considered as control animals A value for the augmentation of the ovarian weight of the experimental animals was obtained by subtracting the average ovarian weight (to the nearest milligram) of the three control animals on that pituitary dosage from the weight of the ovaries of each experimental rat When the weight of the experimental rat s ovaries was equal to or less than the control average an arbitrary value for augmentation of one milligram was assigned This was the case with only three experimental rats

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[1] Supported in part by a grant made from the Wisconsin Alumni Research Foundation

Inasmuch as McShan and Meyer (5) showed that the augmentive action of a sample of blood seemed to be rather completely attributable to its hemin content, another experiment was run in which the plan was similar to that of the first one with the exception that 0, 1, 2, 4, and 8 mgm. dosages of hemin were used instead of the various dosages of blood. The water extract of the pituitary gland was used in this experiment instead of the 33% acetone-soluble fraction of the 50% aqueous pyridine extract.

Two trials were run in this experiment and two rats were run in each trial on each hemin-pituitary extract mixture. In addition to the ovarian weights and the augmentation which was calculated as in the first experiment, a determination was also made of the percentage solids for each pair of rat's ovaries so that its relationship to augmentation could be determined.

Results. The average weight of the ovaries for the control rats on the 25 mgm. equivalent pituitary dosage (table 1) was not distinguishable from that of rats of the same age in the colony which had not received pituitary extract. When sufficient blood was added to this pituitary dosage, the ovarian weight was increased to 57.3 mgm. (on 3.2 cc. blood). Thus the addition of augmenter to the pituitary extract changed the reaction from below the threshold of detection to one that was definitely observable.

TABLE 1

Effects of pituitary and blood dosages upon average ovarian weight

PIT. DOSAGE MGM.-EQUIV.	BLOOD (CC.)					
	0.0*	0.2	0.4	0.8	1.6	3.2
	milligrams, ovarian weight†					
25	12.7	13.0	19.3	16.3	28.3	57.3
50	16.3	20.3	49.0	51.3	78.3	74.3
100	33.3	52.7	58.7	85.0	169.3	157.3
200	44.3	106.7	110.3	116.3	216.7	205.3
400	79.3	122.3	96.0	170.7	209.0	166.7

* Controls.

† All averages are based upon groups of 3 rats.

The other control dosages of pituitary extract produced ovarian responses ranging from barely detectable (16.3 mgm.) to moderately strong (79.3 mgm.). The different experimental groups exceeded their respective control groups in ovarian weight in all cases but the amounts by which they exceeded were variable.

An analysis of this variation[2] in augmentation for all the experimental animals showed that both blood and pituitary dosages produced statistically significant and quantitatively important differences (table 2). A lesser amount of variation, but significant at a probability of 0.05 was due to the interaction of blood and pituitary dosages. This means that the respective differences between pituitary dosages varied between blood dosages and the converse of this, of course, also being true

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[2] According to the analysis of variance procedure of R. A. Fisher as presented by D. D. Paterson (1939), Statistical Technique in Agricultural Research. 263 pp. McGraw-Hill Book Company, New York.

On the hemin experiment, all of the control groups of rats receiving pituitary extract alone gave definite responses, also, the ovarian weights for all experimental groups exceeded those of the respective control groups The rats on the two different trials with hemin are averaged together in table 3, but this is not an entirely satisfactory way of expressing the data because a comparison of the mean square between trials (table 4) with a summation of all interactions with trials, shows the percentages of ovarian solids for the two trials to be significantly different A similar comparison, however, fails to show a significant difference between trials in augmentation

TABLE 2

Analysis of variance in augmentation of pituitary extracts by blood*

CAUSE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F
Blood dosages	4	19571	21 7†
Pit dosages	4	19106	21 2†
Blood × pit	16	1725	1 9‡
Error	50	902	
Total	74	3073	

* Weight of each experimental rat's ovaries minus the average ovarian weight for 3 control rats

† Significant at 1 per cent level of probability

‡ Significant at 5 per cent level of probability

TABLE 3

Effects of pituitary and hemin dosages upon average ovarian weight and average per cent of ovarian solids

PIT DOSAGE MCM EQUIV	MCM HEMIN				
	0*	1	2	4	8
	mgm ovarian wt —per cent ovarian sol ds				
25	17 5—20 6	50 5—18 2†	66 3—18 0	92 8—17 6	102 7—14 9‡
50	39 3—18 6	96 0—16 5	100 8—16 9	213 3—16 4	256 3—14 3
100	85 0—18 4	93 5—18 7	112 3—18 0	201 8—17 4	250 0—15 0
200	114 3—18 2	139 3—17 8	151 0—18 5	177 5—17 8	194 0—16 8
400	122 8—17 8	143 0—17 7	176 8—16 9	162 0—17 5	191 3—16 6

* Controls

† 2 rats only

‡ 3 rats only

All other averages are based on groups of 4 rats

Hemin dosage and pituitary dosage each produced significant variations in the two ovarian characters over the two trials Trial did not affect the respective differences between the different hemin dosages for either ovarian character (augmentation and percentage ovarian solid), but it did interact with pituitary dosage to affect the respective differences in both of the characters. For these reasons, one might expect the variation in both augmentation and percentage ovarian solids due to differences in hemin dosage to be *more predictable* in future trials than he would the differences in these characters due to pituitary dosages.

Just as in the blood experiment, the increases in augmentation due to ascending dosages of augmenter depended upon the dosages of pituitary extract and the converse was also true. The same thing was found to be true for percentage ovarian solids (table 4, hemin x pit.).

The fact that the percentage ovarian solids for the ovaries from rats receiving both hemin and pituitary extract appeared in most instances to be lower than for those rats receiving pituitary extract alone led us to calculate the correlation between ovarian weight and percentage ovarian solids. This was found to be negative and highly significant ($r = -0.70$). This negative correlation describes what is apparent in table 3, that as the differences between the control and experimental ovarian weights increased, the percentage solids appeared to decrease.

TABLE 4

Analysis of variance in augmentation[*] and in per cent of ovarian solids—rats receiving both pituitary extract and hemin

CAUSE OF VARIATION	D/F	AUGMENTATION		PER CENT OF OVARIAN SOLIDS	
		Mean square	F	Mean square	F
Trial	1	9665	3.6	22.74	15.5†
Hemin dosage	3	36098	13.3†	20.20	13.7†
Pit. dosage	4	16017	5.9†	6.34	4.3‡
Interactions with trials	19	2708		1.47	
Hemin × trial	3	1218	1.0	0.74	1.0
Pit. × trial	4	7043	5.6†	2.65	3.7‡
Hemin × pit.	12	4121	3.2†	1.44	2.0‡
Hemin × pit. × trial	12	1636	1.3	1.26	1.8
Error	37	1268		0.71	
Total	76	4340		2.37	

[*] Weight of each experimental rat's ovaries minus the average ovarian weight for 2 control rats.

† Significant at 1% level of probability.

‡ Significant at 5% level of probability.

If a high percentage of ovarian solids indicates a high degree of luteinization, then the important comparison would be that of the ovarian solids in ovaries of the same weight but resulting from augmentation in one case and pituitary stimulation alone in the other. Visual examination gave us the impression that the augmented ovaries were more follicular in character (fewer corpora lutea) than those ovaries from pituitary treatment alone. If one considers only those rats from groups whose averages were within similar weight ranges but from pituitary treatment alone in one case and pituitary plus hemin in the other, he finds three control groups of twelve rats whose averages range from 39.3 to 114.3

mgm. Similarly, he finds 29 rats from groups receiving hemin in addition to pituitary extract and whose eight group-averages range from 50 5 to 112 3 mgm. A comparison of the percentages of solids in these two groups of similar ovarian-weight range shows 18 4 in the control group and 17.4 in the experimental group. The averages for the ovarian weights in these restricted ranges are not entirely comparable (79.5 and 91.5 mgm.) but the percentage solids in the augmented group is less also than that of the rats with heaviest ovaries on pituitary extract alone, which was 17.8% on 122.8 mgm. ovaries.

In neither the blood nor the hemin experiment was the greatest augmentation obtained on either the largest or smallest dosages of anterior pituitary extract in terms of milligram-equivalent (table 1 and table 3). The optimum pituitary dosages for obtaining the maximum augmentation were 100 and 200 milligram-equivalent in the blood experiment, and 50 milligram-equivalent in the hemin experiment. This suggests that there is no relationship between dosage in milligram-equivalent for different extracts and maximum augmentation. The control ovarian weights in the first case were 33.3 and 44 3. In the second case, it was 39.3. Within the limits of these experiments, therefore, it would appear that dosages of pituitary extract yielding average ovarian weights from approximately 33 to 44 mgm. are optimum for obtaining the maximum augmentation.

No such evidence was obtained for an optimum dosage of augmenter within the dosage ranges studied. In the blood experiment there was an increase in augmentation up to 1.6 cc , but no increase between the dosages of 1.6 and 3.2 cc. The very lowest dosage of pituitary extract may have been an exception to this. With the hemin there was no definite indication that the limit of augmentation had been reached between 4 and 8 mgm. dosages.

Unpublished data of this laboratory show that pituitary extracts of the types used give little or no increases above 120 mgm. in ovarian weight by further inincreases in dosage. These data tend to confirm the observations of Deanesly (8) that there was a tendency for ovarian weights to "plateau" at a similar level on pituitary extracts. It is obvious that the pituitary augmenter mixture is capable of producing at least twice this maximum ovarian weight. These large ovaries were composed of corpora lutea and large cystic follicles, and in passing they might be confused from their appearance with the ovaries produced in rats when large quantities of pregnant mare serum are injected.

SUMMARY

The addition of different amounts of "augmenter" (blood or hemin) to pituitary gonadotropic extracts produced variations in the augmentation of ovarian weight. Likewise augmentation by any given amount of augmenter depended upon the dosage of pituitary extract with which it was combined. These determinations were made upon 187 rats, 21 days of age.

The optimum dosages of pituitary extract for maximum augmentation by blood or hemin were those that acting alone produced ovaries weighing 33 to 43 mg. on the average

The relationship of augmentation to percentage ovarian solids was studied in

the "hemin" experiment. The correlation was negative ($r = -0.70; P < 0.01$). The lower percentage of solids in the augmented ovaries appeared to be associated with the presence of fewer corpora lutea and more cystic follicles than in control ovaries of similar weight.

Addition of augmenter to a small dosage of pituitary extract made it possible to develop larger ovaries within a five-day interval than with any dosage of pituitary extract alone.

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STUDIES ON THE DETOXICATION OF ORGANIC ARSENICAL COMPOUNDS

II. Correlation of the Quantity of *p*-Aminobenzoic Acid Required to Protect Rats against High Doses of Carbarsone and Arsanilic Acid

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In the preceding paper of this series (1) it was shown that *p*-aminobenzoic acid, administered as a solution of the sodium salt and hereafter referred to as *p*-aminobenzoate, given parenterally or *per os*, enables rats to survive massive doses (ca. LD_{100}) of carbarsone (*p*-carbamino phenyl arsonic acid) and other phenyl arsonic acid derivatives. In the absence of any rationale on the mechanism of detoxication, at the outset of these studies high doses of *p*-aminobenzoate were deemed necessary to achieve the greatest degree of protection. This assumption was based on two established facts:

(*1*) in the body *p*-aminobenzoate undergoes rapid conjugation (by acetylation (2), union with glycine or other detoxicating agents, (3) etc.) and prompt elimination from the system (4), while

(*2*) the excretion of pentavalent arsenicals, such as carbarsone and 'Tryparsamide', is a protracted process extending over several days (5, 6, 7).

Aiming, therefore, to produce continuous high blood levels of *p*-aminobenzoate, we gave as much as 3 grams per kg. body weight daily by the oral route for 3 to 5 days to the rats.

Although *p*-aminobenzoate, a normal physiological constitutent of the body, has been recognized as a component of the vitamin B complex and is relatively non-toxic on oral administration, when given parenterally (i.-v. or i.-p.) there is a definite limit of tolerance (8). Judging by numerical results, occasionally it appeared that rats on the highest *p*-aminobenzoate dosage did not fare as well as others on a lower dosage Sometimes the injection of *p*-aminobenzoate by the intraperitoneal route caused much discomfort or even sharp pain.[1]

Once the practical possibility of protecting essentially all rats against massive doses of carbarsone, 'Tryparsamide', 'Atoxyl' etc. had been established, it became desirable to investigate the quantitative relationship between the toxic arsonate and the protective *p*-aminobenzoate necessary to obtain optimum results The results of experiments performed with this object in mind are here presented

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[1] This was found to be due to an increase in pH that occurs when solutions of sodium *p* aminobenzoate are allowed to remain exposed to the air for several days All discomfort could be avoided by the use of freshly prepared neutral solutions.

Experimental procedure. As in previous reported studies, simply to facilitate rapid calculation of dosage, rats weighing approximately 100 grams were used when available. They were maintained in communal cages in which balanced commercial diet was provided *ad lib.* and observed for not less than eleven days before any experiment was terminated.

Again, only for the sake of convenience, *p*-aminobenzoate in the form of a freshly prepared 10 or 20% aqueous solution was administered either orally or intraperitoneally, while a solution of the sodium salt of the arsonate was injected some minutes later,[2] usually also into the abdominal cavity.

TABLE 1

Degree of protection conferred by single and multiple doses of p-aminobenzoate against carbarsone and arsanilic acid

DRUG	DOSE (I.-P.)	*p*-AMINOBENZOATE		NUMBER OF RATS TREATED	PERCENTAGE SURVIVAL	
		Dose	Mode of administration		Plus *p*-amino benzoate	Controls
	mgm./kg.	*mgm./kg.*				
Carbarsone	2500	2000 × 2	oral	5	20	
	2000	2000 × 2	oral	5	40	
	1500	3000 × 3	oral	18	94	
		750 × 3	i.-p.	10	100	
		controls		36		0
	1000	750 × 2	oral	10	90	
		controls		54		9.3
Arsanilic acid or 'Atoxyl'	600	1500 × 2	i.-p.	20	35	
	500	1500 × 1	i.-p.	10	90	
	400	2000 × 3	oral	17	100	
		1500 × 2	i.-p.	10	100	
		1000 × 2	i.-p.	10	90	
		1000 × 1	i.-p.	10	100	
		800 × 2	i.-p.	10	90	
		controls		64		12.5

The data incorporated in table 1 are the results of experiments designed to provide information on the maximum dose of two typical phenyl arsonates against which appreciable protection is obtained by the administration of *p*-aminobenzoate.

Without protection, approximately 10% of rats recover after the parenteral injection (i.-p. or i.-v.) of 1000 mgm./kg. of carbarsone or 400 mgm./kg. of arsanilic acid. This dose, then, is taken to be approximately the LD_{90}. The minimum "universal" lethal dose (LD_{100}) of carbarsone is somewhat higher, probably not more than 1200 mgm./kg. At any rate, in our experience not a single rat has survived a dose of 1500 mgm./kg. At this dosage, all animals were protected by the prior administration of *p*-aminobenzoate at the rate of 750 mgm./kg. followed by the same quantity on the two subsequent days. Whether a smaller quantity of *p*-aminobenzoate would have been equally beneficial was not determined. The administration of two daily injections of 2000 mgm./kg. of *p*-aminobenzoate permitted the survival, respectively, of 2 out of 5 rats and of 1 out of 5 rats receiving carbarsone at the rate of 2000 mgm. and 2500 mgm./kg.

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[2] The chronological relation is not an unimportant detail, as will be shown in the next paper of this series.

On slightly smaller doses of *p*-aminobenzoate, namely 1500 mgm./kg., 9 out of 10 rats survived 500 mgm./kg. of arsanilic acid; 7 out of 20 rats survived at the dose level of 600 mgm./kg.

An examination of table 1 will show to what an extent we have on many previous occasions exceeded the minimal quantity of *p*-aminobenzoate necessary to protect all the rats in an experiment. In some instances the repeated injections of large amounts of *p*-aminobenzoate might be interpreted as having had a deleterious influence, but to prove such a contention substantial proof would be required, and the number of rats involved in our experiment is too small to allow for adequate analysis. However, other studies (details of which are reserved for another paper) have clearly demonstrated that unless *p-aminobenzoate is administered within an hour after the injection of the arsenical* there is a decided fall in the degree of protection afforded. After the lapse of a few hours, secondary injections of *p*-aminobenzoate appear to have no detoxicating value; the question of the unfavorable effect of multiple injections of high doses of *p*-aminobenzoate remains open.

In order to determine the lower extreme of the protective range of *p*-aminobenzoate, experiments were set up in which single diminishing doses of *p*-aminobenzoate were administered to several groups of rats that then received injections of carbarsone and arsanilic acid at a rate approximating the LD_{90}, respectively, of these two compounds. The results are presented in table 2.

It is seen from the results of experiments summarized in table 2 that *p*-aminobenzoate at the rate of 500 mgm./kg. is sufficient to confer protection on nearly all rats that receive a carbarsone injection of 1000 mgm./kg. As the "coverage" by *p*-aminobenzoate is reduced below this level, there is a more or less regular decrease in the number of rats that escape death. This regularity in the quantitative relationship is more forcefully revealed when the results are presented in terms of the "average survival time" as computed on a *per diem* basis for the ten day observation period. That even those individual rats that fail to survive have received a partial benefit from a dose of *p*-aminobenzoate insufficient to save their lives is seen when the "average mortality time" is calculated for the various dosage groups.

Against 400 mgm./kg. of arsanilic acid, virtually total protection is afforded by only 250 mgm./kg. of *p*-aminobenzoate. Below this coverage level, one again finds partial protection for the group as well as the individual rat but, probably because of the large time unit (1 day) and the smallness of the groups, the direct relationship between the survival time of the rats that died and size of *p*-aminobenzoate dose is not so clearly demonstrated.

The fact that as small a quantity as 15 mgm./kg. of *p*-aminobenzoate provided for the survival of 5 out of 10 rats is an indication of the remarkable protective value of this substance. Perhaps it also reflects the intrinsic variability of individual rats in their susceptibility to arsenical poisoning and their need for larger amounts of the detoxicating agent.

TABLE 2

Degree of protection afforded by diminishing single doses of p-aminobenzoate against an LD$_{95}$ of carbarsone and arsanilic acid

DRUG AND DOSE	*p*-AMINOBENZOATE DOSE (I.-P.)	NUMBER TREATED	PER CENT SURVIVED	AVERAGE SURVIVAL TIME* (DAYS)	
				Of all rats in group	Of rats that died
	mgm./kg.				
Carbarsone, 1000 mgm. per kg. i.-p.	750	30	86	9.8+	5.7
	500	30	96	9.9+	4.0
	250	19	68	7.9+	3.5
	150	20	25	4.5+	2.7
	100	10	20	3.4+	1.75
	75	10	20	4.1+	2.6
	50	10	0	1.5	1.5
	controls	54	9		1.3
Arsanilic acid, 400 mgm. per kg. i.-p.	1000	10	100	10+	
	600–800	30	86	9.2+	4.0
	500	10	100	10+	
	300–400	21	95	9.9+	9.0
	250	20	95	9.8+	6.0
	200	20	70	7.9+	3.0
	100–150	20	45	6.6+	3.8
	50	10	30	4.6+	2.3
	25	10	50	6.6+	3.2
	15	10	50	5.7+	1.6
	controls	64	12.5		1.4

* These figures represent the average time of survival in days of all rats receiving the specified dosage. Usually where the number of rats involved is more than 10, the figure was derived by averaging the results of two or more experiments. "+" signs signify survival of at least one rat in the group beyond the ten day observation period.

DISCUSSION. In speculating on the possible mechanism whereby such phenyl arsonates as carbarsone and arsanilic acid are detoxicated in the body, because of the similarity of constitution between these compounds we encounter a natural tendency to seek an explanation in terms of the stimulating theory proposed by Woods (9) and others of the English school to account for the specific bacteriostatic effect of the sulfonamide drugs and the phenomenon of strain resistance to these compounds. It will be recalled that, from *in vitro* studies on the growth of streptococci in artificial media, Woods was able to demonstrate that *p*-aminobenzoate (or a substance conforming in chemical characteristics to *p*-aminobenzoate) was an essential requirement for growth and that it was antagonized by sulfanilamide in competition for an enzyme, thus inhibiting growth of the test organism. This "blocking" has with great plausibleness been demonstrated by Fildes (10) and McIlwain (11) to be a consequence of the similarity of chemical configuration In this connection one should also refer to the suggestion by Fildes (12) that *p*-aminobenzoate might neutralize or inhibit the toxic side effects of the sulfonamide drugs, a prospect for which Strauss and Finland (13) could find no clinical confirmation. Some of the most impressive evidence of the rôle played by *p*-aminobenzoate in inhibiting the action of sulfanilamide was derived from the quantitative (molal) relationship of the two substances in their competitive action.

If, by analogy, one were to consider that the protective action of *p*-aminobenzoate is due to its specific ability to "protect" one or more enzymatic actions involved in cell metabolism, enzymes for which the phenyl arsonates have a destructive predilection, the demonstration of a quantitative relationship between these two compounds would constitute strong evidence in favor of the postulate. However, from the data thus far accumulated, it is difficult to determine what quantitative relationship exists between the minimal amount of *p*-aminobenzoate required to neutralize (using the word in a general rather than specifically chemical sense) the action of any specified quantity of pentavalent arsenical. This difficulty is in large part due to the fact that our studies have been concerned with an *in vivo* system in which the effective tissue concentrations of *p*-aminobenzoate and arsenical have not been determined. It is possible that the chances of demonstrating the quantitative relationships by *in vitro* experiments such as the measurement of respiration of tissues in the Warburg apparatus would yield interesting data. On the other hand, the relatively low toxicity of the pentavalent arsenicals, the slowness of their toxic action, and the chemical complexity of the medium in which animal tissues are grown may preclude the sort of *in vitro* experiments that have been so fruitful in revealing the relationship between growth promotors and growth inhibitors in the bacterial kingdom.

SUMMARY

1. By the administration of sufficient *p*-aminobenzoate, essentially all rats may be protected against a "Universal Lethal Dose" (LD_{100}) of such phenyl arsonates as carbarsone and arsanilic acid (= 'Atoxyl'). Beyond this arsenical dose level, there is a gradual decrease in the group survival rate.

2. A single injection of *p*-aminobenzoate is capable of conferring the protection.

3. Against the LD_{90} quantity of these pentavalent arsenicals, protection is afforded by a relatively smaller dose of *p*-aminobenzoate; 15 mgm./kg. was found sufficient to protect 50% of individual rats and to prolong the survival time of a group of rats receiving 400 mgm /kg of arsanilic acid.

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STUDIES ON THE DETOXICATION OF ORGANIC ARSENICAL COMPOUNDS

III The Time Factor Influencing p Aminobenzoate Protection of Rats Receiving Lethal Doses of Phenyl Arsonates

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As shown in the preceding paper of this series (1), the administration by the intraperitoneal route of 500 mgm /kg of sodium *p* aminobenzoate counteracts the lethal effect of (*a*) 1000 mgm /kg of carbarsone (*p* carbamino phenyl arsonic acid) or (*b*) 400 mgm /kg of arsanilic acid (*p* amino phenyl arsonic acid or 'Atoxyl') Occasionally, in our early experiments, even higher doses of *p*-aminobenzoate did not provide complete or "overall" protection, from 2 to 5 rats in a group of 10 eventually succumbing An investigation designed to reveal some of the causes underlying the failure to impart protection to all individual animals on what appear to be adequate doses of *p* aminobenzoate forms the subject of the present communication

On analyzing protocols and checking on dosage calculations etc , it was noted that less effective protection was more likely to occur when work involving a large series of rats was undertaken than when an experiment called for a moderately small number of animals Further, it was observed that the outcome was likely to be less favorable when the entire series of rats was first injected with the arsenical, in consequence of which a considerable period might elapse before the *p* aminobenzoate was administered

While providing us with necessary information on factors that might influence the efficacy of protection, it appeared, incidentally, that this study might shed some light on the mechanism underlying the detoxicating action of *p* amino benzoate

Experimental procedure Conditions under which these experiments were performed were essentially the same as outlined in our earlier papers Groups of 10 or more unselected albino rats of 90 to 110 grams weight were injected intraperitoneally with either carbarsone or arsanilic acid in 20% aqueous solution (of the sodium salt) at the approximate LD_{50} rates of 1000 and 400 mgm per kg respectively The arsenical injection either preceded or followed at various timed intervals the injection of *p* aminobenzoate For the control series, either the calculated dosage of *p* aminobenzoate and arsonate was mixed and given as a single injection or not more than a few minutes were allowed to intervene between the injection of the separate solutions Experimental animals were in all instances observed for at least eleven days In an isolated case or two on account of the present difficulty in securing an adequate supply of rats commercially, we have, after a short rest interval, reused rats that had previously been subjected to protection tests Such survivors are capable of living the normal rat life span, and we have never observed that, as a result of their previous experience, these were either more or less susceptible to arsenic or less amenable to protection by *p*-aminobenzoate.

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[1] With acknowledgment to Mrs Nina Belle Boling and Mr Charles R Hamilton for valued technical assistance in the performance of this study

In order to eliminate superfluous variable factors, it was decided that the protective dose of *p*-aminobenzoate should be uniform and at the rate of 750 mgm./kg. This quantity of *p*-aminobenzoate is well within the limits of tolerance and yet is from one and one-half to three times the quantity previously found sufficient for the protection of nearly all rats in groups receiving the LD_{50} of carbarsone or arsanilic acid (2).

From the results of experiments abstracted in tables 1 and 2 it is seen that, on the average, the outcome of a protection experiment is decidedly influenced by a time factor. "Neutralization" of the lethal effects of the arsenical is fully achieved when both *p*-aminobenzoate and arsenical are administered simultaneously (mixed) or when *p*-aminobenzoate is administered within one hour (in the case of carbarsone) or up to three hours (in the case of arsanilic acid) *in advance* of the arsenical. On the other hand, should the arsenical be injected first, as short an interval as 15 minutes (for carbarsone) and 30 minutes (for arsanilic acid) is associated with a significant reduction in the detoxicating potentialities of *p*-aminobenzoate. A greater lapse of time is accompanied by an almost directly proportional loss of protection.

DISCUSSION. If speculation as to the mechanism underlying detoxication of the pentavalent arsenicals may be initiated on the basis of evidence thus far presented, considerable significance, we believe, must be attached to the results recorded in the present paper. In our opinion, no rationale can be acceptable unless it satisfactorily accounts for the cumulative effect of the time factor whose influence on the degree of protection, even when this is measured by so crude a yardstick as "percentage of group surviving", shows remarkable regularity.

The fact that *p*-aminobenzoate bears a similar structural relationship to arsanilic acid as it does to sulfanilamide suggests that one might find an explanation of detoxication on lines parallel to the enzyme blockade theory that is currently receiving wide acceptance as a basis whereby the mechanism of bacteriostasis by sulfonamide drugs can be rationalized. Among other sources of supporting evidence, this theory has received confirmation from the demonstration of a strict quantitative relationship between "essential metabolite" (*e.g.*, *p*-aminobenzoate in medium for bacterial growth *in vitro*) and its "inhibitor" (*e.g.*, sulfanilamide) (2), from the studies of Keltch *et al.* (3, 4) on the effect of certain local anesthetics, and from the more recent work of McIlwain (5).

It should be unnecessary to point out that the present studies have been concerned entirely with an *in-vivo* system. Though there appears to be no indication even of an approximate equivalence in moles of the quantities of *p*-aminobenzoate found necessary to inhibit the lethal result of various quantities of the different arsenicals, the actual molar relationships required to achieve "inhibition" of the lethal effect could only be revealed from a quantitative determination of the critical blood and tissue levels of the two antagonistic compounds in rats that survive as compared with those that succumb.

TABLE 1

Influence of sequence and time interval on protection of rats 1000 mgm /kg carbarsone i p , preceded or succeeded by 750 mgm /kg p-aminobenzoate i p

SEQUENCE	TIME INTERVAL	NUMBER ANIMALS USED	PERCENTAGE SURVIVED	AVERAGE SURVIVAL PERIOD (DAYS)	
				For group	For rats that died
	minutes				
p aminobenzoate *prior to* carbarsone	360	10	10	3 2+	2 4
	240	10	50	7 8+	5 6
	120	10	70	7 9+	3 0
	60	18	100	10+	
	30	20	100	10+	
	15	15	93	9 5+	7 0
p aminobenzoate plus carbarsone	simultaneous	25	100	10+	
Carbarsone alone	control	56	9 0		1 3
Carbarsone *prior to* *p* aminobenzoate	15	15	73	8 5+	3 7
	30	20	10	2 5+	1 7
	60	20	55	7 0+	3 5
	120	10	10	2 0+	0 6
	240	10	10	2 5+	1 1
	360	10	0	1 0	0 5

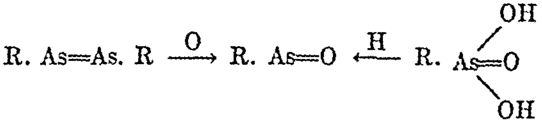
TABLE 2

Influence of sequence and time interval on protection of rats 400 mgm /kg arsanilic acid i p , preceded or succeeded by 750 mgm /kg p aminobenzoate i p

SEQUENCE	TIME INTERVAL	NUMBER ANIMALS USED	PERCENTAGE SURVIVED	AVERAGE SURVIVAL PERIOD (DAYS)	
				For group	For rats that died
	minutes				
p aminobenzoate *prior to* arsanilic acid	540	10	20	4 4+	3 0
	450	10	50	6 3+	2 6
	400	10	60	8 1+	5 2
	300	10	70	7 9+	3 0
	180	10	100	10+	
	60	10	100	10+	
	45	10	80	8 2+	0 5
	30	10	100	10+	
p aminobenzoate plus arsanilic acid	simultaneous	25	96	9 7+	4 0
Arsanilic acid alone	control	68	9 3		2 3
Arsanilic acid *prior to* *p* aminobenzoate	30	20	90	9 5+	4 5
	45	20	55	6 3+	1 7
	60	10	50	5 5+	1 4
	120	10	20	3 4+	1 8
	180	10	40	4 8+	1 3
	240	10	30	4 0+	1 4
	300	20	0	1 0	0 9

Unfortunately the exigencies of war preclude the chemical studies necessary for the acquisition of these essential data at present. Nevertheless it may be useful to speculate on the mechanism of this detoxication phenomenon from another angle. It may not be remiss to point out that our publications up to this time have been devoted to the detoxication exclusively of pentavalent phenyl arsonates and stibonates (6) by *p*-aminobenzoate. Studies still in progress have revealed a number of other compounds, some not even remotely related to *p*-aminobenzoate, which have detoxicating value against the pentavalent arsenicals. On the other hand, we have found that *p*-aminobenzoate does *not* confer much protection against lethal doses of the trivalent arsenicals, more especially against *m*-amino *p*-hydroxyphenyl arsenoxide ('Mapharsen').[2] This, we suspect, is of special significance, for it fits in with what has been learned about the time relationship.

It is well known that neither the pentavalent arsenicals nor the trivalent arsenobenzenes are as immediately toxic for mammals or as directly parasiticidal for trypanosomes as are the corresponding "arsenoxides" drived by reduction or by oxidation according to the scheme:



$$R.\,As{=}As.\,R \xrightarrow{O} R.\,As{=}O \xleftarrow{H} R.\,As(OH)_2{=}O$$

On injecting a lethal dose of "arsenoxide" into a rat, symptoms of poisoning appear within a few minutes, and the animal dies oftentimes in less than an hour. In contrast, a lethal dose of pentavalent arsenicals rarely produces immediate signs of intoxication, and often more than 24 hours will elapse before the rat succumbs. Reflecting the same basic toxic process is the well established fact (7) that trypanosomes commence to disappear from the blood of infected rats almost immediately on injecting "arsenoxide", as compared with a latent period up to six hours after the injection of an effective dose of arsphenamine, or 12 to 18 hours after such a pentavalent arsenical as 'Arsacetin' (*p*-acetylaminophenyl arsonic acid).

It is reasonable to account for this latent period by identifying it with the time required for the reduction of sufficient pentavalent arsenical to attain the toxic threshold of "arsenoxide". The formation of "arsenoxide" in animals after the injection of arsphenamine has actually been demonstrated by Rosenthal (8). According to Warburg (quoted by Rosenthal and Voegtlin (9)) the poisonous effects of arsenicals are due to the chemical action of arsenious acid on an iron-containing respiratory enzyme. Voegtlin, Dyer and Leonard (10) are of the opinion that "arsenoxide" exerts its action on mammalian protoplasm through its chemical affinity for the sulphydryl group of certain proteins (*e.g.*,

[2] Unreported studies from this laboratory show a definite order of protection only against neoarsphenamine.

glutathione), leading to the death of cells by asphyxiation. The destruction of trypanosomes by "arsenoxide" is believed to take place through a similar sulphydryl mechanism. At any rate, it was shown by Voegtlin and Dyer (11) and by Voegtlin, Dyer and Leonard (10) that the injection of such an —SH compound as reduced crystalline glutathione counteracts the toxic effect of arsphenamine on the host and diminishes its therapeutic (parasiticidal) effect on the trypanosomes.

In previous papers (6, 12) we have presented evidence that even very high doses of p aminobenzoate fail to inhibit the trypanocidal action of either pentavalent arsenicals or antimonials.[3] It may, therefore, be safely inferred that p-aminobenzoate does not appreciably interfere with the reduction process whereby the trypanocidal "arsenoxide" derivative is produced in the body.

Taken in conjunction with the above mentioned finding, that p aminobenzoate offers little protection against "arsenoxide", it is difficult to regard as purely fortuitous the fact that the time required for the conversion of the relatively innocuous pentavelent arsenical into the directly trypanocidal and host-toxic "arsenoxide" almost coincides with the time found for p-aminobenzoate to commence losing its protective properties when introduced into the body prior to the arsenical.

The survival rate of rats that receive p-aminobenzoate before the arsenical may well be determined by the rate of p-aminobenzoate excretion as worked out by Quick (13) in the dog and by Strauss *et al.* (14) in man, reciprocally, it may also be an index of the speed with which "arsenoxides" are produced in the body by reduction of the various pentavalent arsenical compounds.

On the basis of other studies, it is felt that it would be premature at this stage to continue further speculation on the mechanistic aspectsof arsenical detoxication. It is clear that much work of a biochemical nature, particularly with respect to the part that p-aminobenzoate plays in the normal metabolism of the cell and its enzyme systems, is called for before any satisfying theory can be elaborated.

SUMMARY

1. The time factor entering into the sequence of administering uniform doses of p-aminobenzoate and either of two typical phenyl arsonic acid derivatives to rats is found significantly to influence the survival rate of the group

2 The prior injection of p-aminobenzoate up to three hours before the arsenical confers protection upon nearly all animals In contrast, the injection of the arsenical thirty minutes *before* the p aminobenzoate is associated with a distinct reduction in the group (or overall) protection, and this reduction proceeds *pari passu* with the increase in the time interval

3. An explanation of this phenomenon in terms of the *in-vivo* reduction of pentavalent arsenicals to the trivalent "arsenoxides" is tentativelypresented.

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[3] Nor does it interfere with the trypanocidal action of neoarsphenamine, according to findings in this laboratory

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THE EFFECT OF SODIUM DIPHENYL HYDANTOINATE (DILANTIN SODIUM) ON THE UTILIZATION OF ASCORBIC ACID BY GUINEA PIGS[1]

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It has been reported that sodium diphenyl hydantoinate (dilantin sodium), an effective anticonvulsant agent in epilepsy, may stimulate gingival hyperplasia in some patients when given over extended periods. Kimball (1, 2) associated this with a possible vitamin C deficiency. Frankell (3) made similar observations and reported some evidence that the plasma ascorbic acid level was lowered. Drake, Gruber and associates (4, 5) claimed that dilantin sodium definitely lowered the vitamin C of the blood in guinea pigs and also of the tissues in rats. An opposite view has been expressed by Gruhzit (6), Merritt and Foster (7), Hawke (8), Ziskin *et al.* (9), and Millhon and Osterberg (10). This report is further evidence in support of the latter opinion.

The plan of our study included feeding young guinea pigs a basal scurvy-producing diet, then giving them graded amounts of ascorbic acid and, in turn, supplementing this with different doses of dilantin sodium. The criteria used were (*1*) the effect on weight increment, (*2*) gross pathological changes, (*3*) assay for the ascorbic acid plasma levels, and (*4*) the determination of the ascorbic acid content of the adrenals, brain, testes and liver.

EXPERIMENTAL. *Animals and diet.* Young guinea pigs weighing 250–300 grams were kept in groups of 4 to 11 animals each. The scurvy diet fed throughout was essentially that of Sherman (11). It consisted of rolled oats, 29.5%, wheat bran, 29.5%, heated skim milk powder, 30%, butter fat, 9%, cod liver oil, 1% and sodium chloride, 1%. In addition, water and autoclaved clover hay were given *ad libitum*. The animals were weighed at regular intervals and, as the experiment progressed, observations were recorded on the appearance and condition of the fur, eyes, teeth, gums, tenderness and enlargement of the joints. Further, on autopsy, the following were noted: firmness of teeth, presence of "rosary" on the ribs, enlargement of joints, hemorrhagic appearance of the knees, elbows and intestinal tract. The lungs, liver, kidneys and adrenals were also observed.

Experiments. Four feeding experiments were carried out. In each, the animals were divided into 5 to 7 groups, some of which served as controls while, with the other groups, the interrelationship of ascorbic acid and dilantin sodium was studied. Experiments 1 and 2 were the preventive scurvy type and experiment 3 was the curative type. In these, the weight increment, the general appearance and autopsy findings were noted. In experiment 4, preventive type, the ascorbic acid plasma levels and the ascorbic acid content of tissues were determined.

Methods. In experiments 1, 2 and 3 the ascorbic acid was given orally by pipette and the dilantin sodium orally from capsules by emptying the powder well back in the throat and following with a few drops of water. In experiment 4 the ascorbic acid was administered subcutaneously, the concentration of the solution was such that the dose was not more than 1.0 cc. The dilantin sodium was given in the same manner as in experiments 1 and 2.

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[1] Presented before the September 1942 meeting of the American Chemical Society, Buffalo, N. Y.

The samples for the blood plasma were taken by cardiac puncture from each guinea pig once a week, over a period of 7 weeks. Further, the actual time of drawing the test sample was adjusted so that it fell between 16 and 20 hours after administering the supplements. To this end, the animals from each group were always handled routinely in the same order. Immediately after taking the blood and transferring it to tubes, it was centrifuged. The plasma ascorbic acid was determined at once by the Farmer-Abt (12) micro-method.

For the tissue work, male guinea pigs were taken. They were killed by decapitation. Following the Bessey technic, (13) the tissues (the adrenals, brain, right testis and liver) were handled as rapidly as possible, weighed, ground with washed sand in the presence of 3% metaphosphoric acid, diluted with more acid, centrifuged and assayed colorimetrically, using the Evelyn photocolorimeter.

TABLE 1

Effect of dilantin sodium on weight increment

GROUP	DAILY TREATMENTS		NUMBER OF ANIMALS	BODY WEIGHT		GAIN
	Ascorbic acid (a)	Dilantin (b)		Initial	Final	
Experiment 1 (7 weeks)						
	mgm.	*mgm.*		*grams*	*grams*	*grams*
I	Lettuce	None	4	261	545	284
II	Lettuce	50	4	258	479	223
III	0.5	None	4	249	456	207
IV	0.5	50	4	264	498	234
V	0.5	100	4	258	479	223
Experiment 2 (7 weeks)						
I	Lettuce	None	5	308	544	236
II	Lettuce	50	7	284	502	218
III	0.5	None	8	308	525	217
IV	0.5	50	9	296	487	191
V	0.5	100	7	282	475	193
VI	None	None	9	316	196(c)	−120

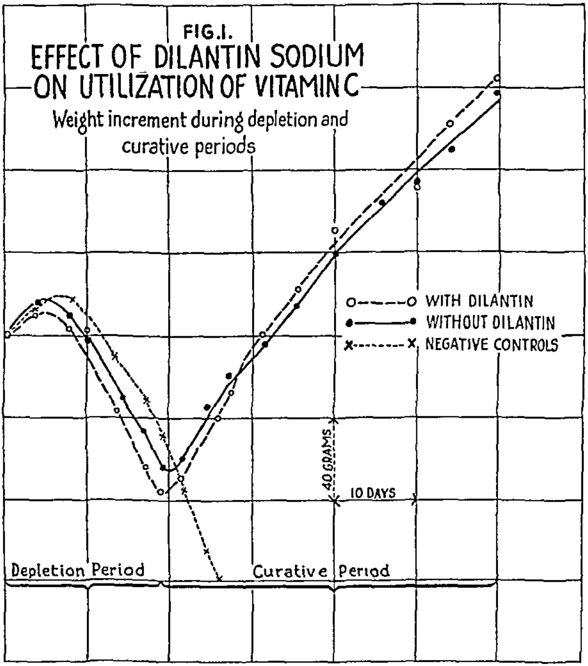
(a) Mgm. per guinea pig per day.
(b) Mgm. per kilo. per day.
(c) Weight on 26th day.

RESULTS. *Effect on weight increment:* The average weight gains for experiments 1 and 2 are given in table 1 and those for experiment 3 in figure 1. The data are arranged primarily so as to compare the effect of the two supplements, ascorbic acid and dilantin sodium. The level of vitamin C (0.5 mgm. daily) was intentionally kept low while that for the dilantin sodium was very high. It was thought that such a high ratio would tend to accentuate any effect that dilantin sodium might have.

The results indicate, except for group VI, the negative control, that all the guinea pigs made relatively good gains. All animals were active and alert and gave no outward indications of having any scurvy symptoms.

Specifically, the data in both experiments 1 and 2 indicate for groups I and II (lettuce with and without dilantin sodium) that the drug seemed to retard the gains. This difference was due in part however to variations in animals, and in part to the handling which the guinea pigs of group II received in their daily treatment. Comparing the values in groups III, IV and V, the evidence indicates that dilantin sodium had very little effect.

Additional data in experiment 3 (fig. 1) substantiate this. Here there were three groups, having 9 to 11 animals each. All were kept on the vitamin C depletion diet for about three weeks in order to permit scurvy to develop (as measured by weight). One group received 50 mgm. dilantin sodium per kilogram per day while the other two had no supplement. From this time on, one group was continued on the scurvy diet as negative control and two groups (one with and without dilantin sodium) were given, over a curative period of 45 days, 0.5 mgm. of ascorbic acid per day per pig. It will be seen from the curves that



the animals receiving the dilantin sodium behaved no differently than the controls throughout the depletion and curative periods. Further, examination upon autopsy of these guinea pigs showed no differences between the groups with respect to symptoms of scurvy including gingivitis.

Effect on the plasma ascorbic acid levels: Our original plan in experiments 1, 2 and 3 called for observations on the plasma ascorbic acid level. We found, however, that the normal plasma level was so very low (0.02 to 0.05 mgm. %) that it would be difficult to measure any significant differences in a decline. Later, Drake and associates (5), in studying this same problem with guinea pigs, apparently overcame the difficulty by first giving the animals an excess of ascorbic acid (5 mgm. per day). In this manner they raised the vitamin C blood level to around 0.6 mgm. %. Under these conditions they found, upon giving 13 mgm. of dilantin sodium per kilo per day, that the ascorbic acid level dropped to around 0.3 mgm. % and on withdrawing the drug, it came up to 0.6–0.7 mgm. Unfortunately the authors did not state how soon the blood samples were taken following their treatment with ascorbic acid and dilantin sodium, nor did they give any data for control groups. Since it is known that plasma vitamin C levels drop very rapidly immediately after giving ascorbic acid, the obvious question arises as to whether Drake *et al.* made their observation before the animals had time to allow the blood vitamin C level to approach a constant or maintenance phase. This rapid decline was shown by Zilva (14) who found the plasma ascorbic acid level after injecting 50 mgm. of vitamin C was 32.5 mgm. % in 30 minutes and down to 3.5 mgm. in 4 hours.

In experiment 4, we followed the plan of Drake *et al.* in raising the ascorbic acid plasma levels above that of the controls. Our procedure differed from theirs fundamentally, however, in three respects: First, the basal rations were not alike, second, Drake *et al.* studied the whole blood while we tested plasma; and third, we adjusted the time of taking the cardiac puncture samples so as to avoid the rapid falling phase. We therefore allowed appreciable time for the ascorbic acid blood levels to approach maintenance. Following our daily routine sequence of carrying out the various steps in the technic, this interval of time was 16–20 hours.

The data are presented in table 2. Groups I and II serve as the controls and give values of 0.01–0.03 mgm. ascorbic acid per 100 cc. of plasma. Group III and IV correspond with those of Drake *et al.* It will be seen that while the vitamin C levels are definitely higher than the controls they are decidedly lower than theirs. When the data are examined with respect to dilantin sodium, they indicate that the drug had no effect. In order to raise the so-called maintenance plasma level further, in group V, VI and VII, 25 mgm. of ascorbic acid were injected subcutaneously. Besides, in group VII, the daily dose of dilantin sodium was increased from 13 to 50 mgm. per kilo per day, which is 10 times the human daily dose. It will be seen that while the plasma levels were thus raised to around 0.4 mgm. %, the values for the different groups were practically identical, indicating again that dilantin sodium had no effect on the utilization of vitamin C.

Effect on the ascorbic acid content of tissues: Since we were unable to find any effect of dilantin sodium on the plasma ascorbic acid levels, we decided to investigate its effect on the storage of this vitamin in the tissues of the animals Determinations were accordingly made on the adrenals, brain (cerebrum), liver

TABLE 2

Effect of dilantin sodium on plasma ascorbic acid

GROUP	NUMBER OF ANIMALS	TREATMENT		AVERAGE MILLIGRAMS ASCORBIC ACID PER 100 CC PLASMA							
		Ascorbic acid (a)	Dilantin (b)	Preliminary period	Experimental Period						
					1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
I	25	Lettuce	None	0 05	0 02	0 01	0 01	0 02	0 02	0 03	0 03
II	17	None	None		0 02	0 02	0 02	0 01	0 03	0 01	
III	16	5	None	0 18	0 14	0 14	0 18	0 14	0 12	0 13	0 11
IV	24	5	13	0 19	0 15	0 13	0 12	0 12	0 11	0 11	0 10
V	20	25	None	0 51	0 36	0 36	0 31	0 27	0 31	0 32	0 33
VI	29	25	13	0 40	0 35	0 35	0 30	0 27	0 33	0 32	0 28
VII	25	25	50	0 40	0 32	0 34	0 31	0 26	0 32	0 32	0 30

(a) Mgm per guinea pig per day
(b) Mgm per kilo per day

TABLE 3

Effect of dilantin sodium on ascorbic acid content of tissues

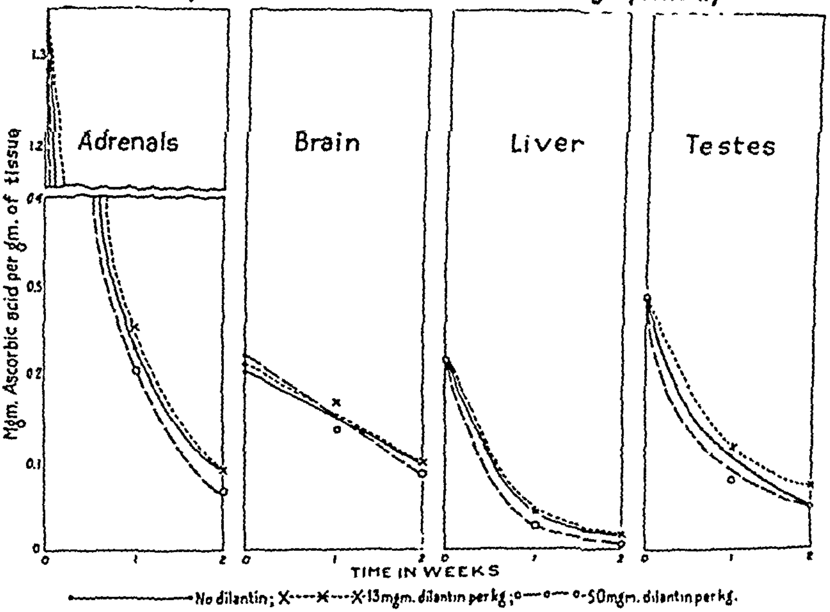
GROUP	TREATMENT		ADRENALS			BRAIN			TESTIS			LIVER
	Ascorbic acid (a)	Dilantin (b)	Gms tissue two adrenals	Mgm per gm of tissue	Total mgm in adrenals	Gms tissue	Mgm per gm of tissue	Total mgm in brain	Gms tissue right testis	Mgm per gm of tissue	Total mgm in testis	Mgm per gm of tissue
I	Lettuce	None	0 376 ±0 056(c)	0 128 ±0 022	0 046 ±0 008	3 15 ±0 20	0 044 ± 006	0 137 ±0 015	2 38 ±0 09	0 041 ±0 005	0 097 ±0 009	0 076 ±0 006
III	5	None	0 404 ±0 04	0 686 ±0 064	0 265 ±0 013	3 07 ±0 07	0 185 ±0 005	0 569 ±0 018	2 32 ±0 16	0 148 ±0 014	0 335 ±0 020	0 102 ±0 008
IV	5	13	0 527 ±0 076	0 582 ±0 047	0 300 ±0 078	3 16 ±0 08	0 173 ±0 011	0 546 ±0 034	2 40 ±0 14	0 139 ±0 009	0 333 ±0 012	0 092 ±0 010
V	25	None	0 413 ±0 020	1 351 ±0 088	0 563 ±0 060	3 14 ±0 08	0 207 ±0 004	0 649 ±0 071	2 47 ±0 07	0 289 ±0 007	0 700 ±0 012	0 217 ±0 016
VII	25	50	0 546 ±0 053	1 356 ±0 031	0 743 ±0 074	3 08 ±0 03	0 218 ±0 004	0 670 ±0 003	2 27 ±0 17	0 285 ±0 011	0 647 ±0 053	0 219 ±0 008

(a) Mgm per guinea pig per day
(b) Mgm per kilo per day
(c) ± Values represent standard error

and testes of the animals used in the plasma study The values obtained are shown in table 3 In no case is there a significant difference between the corresponding values for the ascorbic acid content of the tissues of the dilantin sodium treated animals and their controls. A difference which approaches statistical significance exists for the mgm. of ascorbic acid per gram of adrenal gland between groups III and IV. Further inspection of the data shows this to be due to a slight hypertrophy of the adrenals of the dilantin sodium treated animals, and that actually the total amount of ascorbic acid stored in the adrenals is greater than that of their controls. It is interesting to note that the low level of ascorbic acid in the tissues of the normal control animals, group I, is in good accordance with the low values for plasma ascorbic acid (table 2).

At the end of this stage of our study of the vitamin C content of the tissues we discontinued the injection of ascorbic acid supplement of the remaining animals in groups V, VI and VII, and continued the daily treatments with dilantin sodium to see if some difference might arise from the effect of depletion. At the end of one week and again after 2 weeks on the vitamin C-free regime, analyses were made of the ascorbic acid content of the tissues. Three animals from each group were taken, males being selected due to the fact that most of the females were either pregnant or nursing their young. In this way we were able to observe the effect of the drug on tissue levels far below saturation. The results are shown in figure 2. Examination of the graphs reveal that there is practically no difference between the curves obtained for the dilantin-treated animals and their controls. The curves for the group receiving the higher dosage of dilantin

Fig. 2. Effect of Dilantin Sodium on Vitamin C Utilization. (Ascorbic Acid Content of Tissues During Depletion.)



run a little below the curves for the other two groups in every case, but this difference is much too small to be considered significant. The striking difference between the slope of the curve obtained for the brain as compared with the curves for the three other tissues seems worthy of note. The data of table 3 show that this organ is also the first of the four to become saturated with ascorbic acid when this vitamin is available to the animal. The physiological significance of this finding remains to be pointed out

SUMMARY AND CONCLUSIONS

In a study of the influence of sodium diphenyl hydantoinate (dilantin sodium) on the utilization of vitamin C by young guinea pigs, observations were made on the gain in weight, gross pathological changes, plasma vitamin C levels and the ascorbic acid content of tissues

Feeding experiments were carried out with both the curative and preventive vitamin C regime The Sherman basal scurvy ration was used, supplemented in one series with a minimum oral daily dose of 0 5 mgm of ascorbic acid. Dilantin sodium was then given orally to some groups in massive amounts—50 and 100 mgm per kilo (10 to 20 times the human dose) After 7 to 9 weeks the guinea pigs were autopsied In another series (prophylactic) guinea pigs were given daily oral doses of 13 and 50 mgm of dilantin sodium per kilo and after 1 to 2 hours injected subcutaneously with 5 and 25 mgm. of ascorbic acid per pig per day, over a period of 8 to 14 weeks.

The results indicate: (*a*) that the dilantin sodium had little or no effect on the gain or loss in weight, (*b*) that the dilantin sodium treated guinea pigs were able to breed and rear their young, (*c*) that dilantin did not raise or lower the plasma ascorbic acid levels, (*d*) that the drug had no influence on the ascorbic acid content of the adrenals, brain, testes and liver, and (*e*) that there were no gross pathological manifestations of scurvy including gingivitis

From the above findings we feel justified in concluding that sodium diphenyl hydantoinate (dilantin sodium) has no significant effect on the utilization of ascorbic acid by the guinea pig

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STUDIES ON THE CHEMISTRY AND PHARMACOLOGY OF THE MELANOPHORE HORMONE OF THE PITUITARY GLAND[1]

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In recent reports from this laboratory, there have been presented two important observations on the chemistry of the melanophore hormones (*a*) an increase in potentiation of the hormone with change in pH from low to high values, and (*b*) a difference in pharmacological properties between the "potentiated" and the "unpotentiated" substance. This led us to believe that potentiation of the hormone is due to an effect on the molecule by changes in pH. Therefore, in the assay of the melanophore material, a standard should be prepared at the same pH as that of the unknown (1, 2).

In the present paper, further experimental evidence will be given to support the above statements. In the light of the new findings, two methods will be offered for the assay of the melanophore substances.

EXPERIMENTAL METHODS. Hypophysectomized frogs (*Rana pipiens*) weighing 20 to 30 grams were employed as test objects. The technique of hypophysectomy is very simple and has been described elsewhere (3). The melanophores were examined under a binocular microscope, using 48 mm. objective and 10x oculars. The cells of the webbing of the foot were found to be most constant and uniform in their response to melanophore substance, provided the legs were not affected by diseases such as "red leg." Only hypophysectomized frogs in which the melanophores were fully contracted to circular or oval shapes were selected for quantitative studies. A gradual increase in sensitivity to melanophore substance was observed for some days following hypophysectomy; therefore only animals which had been operated on the same day or within an interval of three days were chosen for each experiment.

Injections were made into the ventral lymph sac of the frog through the mouth. In the assay procedure the same volume of solution, usually 0.25 cc., was used.

The activity of a melanophore preparation was determined by the total response of the melanophores, for which we adopted the length of time which is required for the melanophores to expand and to return to full contraction after injection (1). The response of the melanophores in the entire web of one foot was observed repeatedly. For each determination four frogs were employed. The melanophore activity was calculated from the average value of total responses of four frogs.

The effect of hydrogen ion concentration on potentiation was investigated over a wider range of pH values than that previously employed. Instead of extracting a standard powder of the posterior pituitary with solution of different pH by boiling two minutes and filtering (1), a standard solution of the posterior pituitary, prepared according to the procedure given in U.S.P. XI, was added to solutions of differing pH, and boiled for three minutes. The three-minutes' boiling of 10 cc. solution was conducted in a 30 cc. flask covered with a boiling cap. When a longer period of boiling was desired, the solution was heated in a flask with a reflux condenser.

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To determine the rate of potentiation and destruction of the melanophore substance, the reaction was studied in an alkaline medium at various temperatures. The general procedure is that ordinarily used for studying the velocity of reactions. At different times of the reaction, 1 cc. of the reacting medium was withdrawn and added to 9 cc. of acetic acid of such strength as to neutralize the amount of alkali. This was then immediately assayed in hypophysectomized frogs.

Further details of the procedure for the estimation of the melanophore substance will be given in the section on the methods of assay.

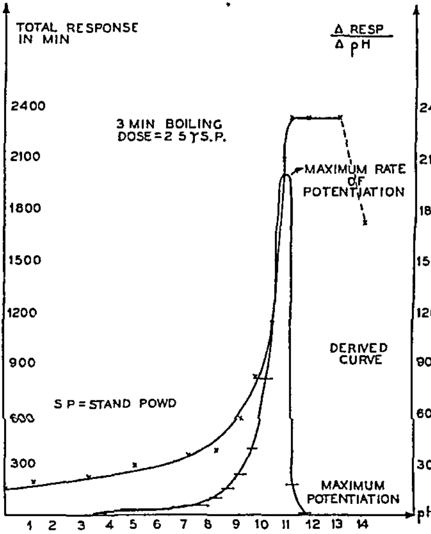

FIG 1

RESULTS 1. *The effect of pH on potentiation.* In figure 1, the pH values are given on the abscissa and the total response in minutes or the change of total response with pH is indicated on the ordinate. The derived curve was obtained by graphical differentiation. It will be seen from the pH-response curve that, after three-minutes' boiling, there is a gradual augmentation of the melanophore activity as the pH rises from 3.25 to 9. Higher than pH 9, this increase of activity with pH becomes very rapid and reaches a maximum at pH 11.2. No more increase of melanophore activity occurs as the pH is further increased to 13. At pH 14, on the other hand, the activity is considerably lessened. This signifies that pH 11.2 and three-minutes' boiling are the optimal conditions for maximal potentiation of the melanophore hormone.

The characteristics of the pH-response curve suggests the possibility that the maximum potentiation may be obtained also at a pH lower than 11.2, mainly by increasing the duration of boiling. Data bearing on this conjecture are presented in figure 2 and table 1. The graphs in figure 2 show that, although potentiation increases with the duration of boiling, an equilibrium condition is established eventually, after which no further potentiation takes place. At equilibrium, only a certain percent of the hormone is potentiated, increasing with the rise of pH. As indicated by the data in table 1, the fraction of the unpotentiated hormone may be further potentiated by the increase of pH of the medium.

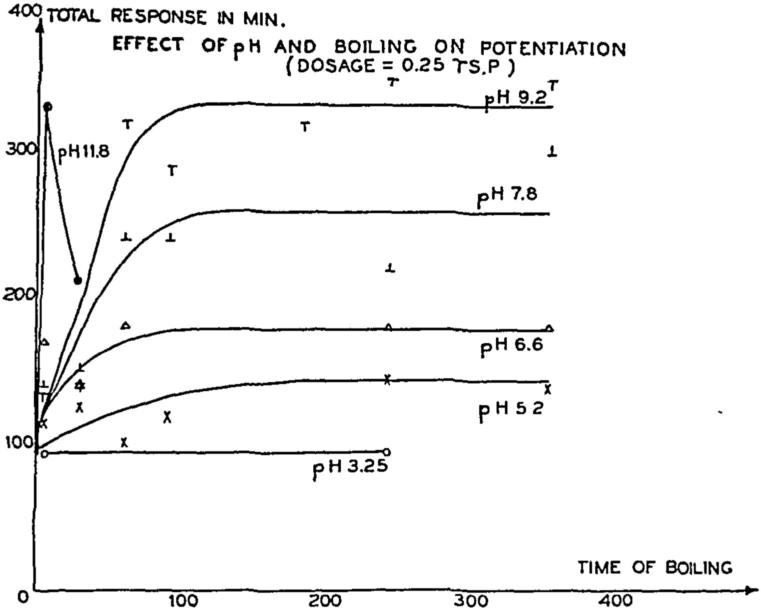

FIG. 2

By taking the values of total response from the plateau of the curves in figure 2, which correspond to the maximal activity of a melanophore preparation potentiated at that pH by long boiling, and plotting them against the respective pHs, the pH-response curve in figure 3 is obtained. From these two graphs it may be seen that the hormone may be completely potentiated at pH 11.2 after three-minutes' boiling or at pH 9.2 after 2 hours. However, owing to an equilibrium condition established between the unpotentiated and the potentiated substance at a pH lower than 9, potentiation does not undergo completion even after prolonged boiling.

Another point of interest may be observed from the derived curves in figures 1 and 3 The maximal increase in potentiation with pH after three minutes' boiling is at pH 10 8 (fig 1), after prolonged boiling it is at pH 7 2 (fig 3)

TABLE 1

pH	TIME OF BOILING	TOTAL RESP	CHANGED TO pH	TIME OF BOILING	TOTAL RESP
	hours	*minutes*		*minutes*	*minutes*
3 3	4	80	11 8	3	510
5 0	4	100	11 8	3	420
7 2	4	180	11 8	3	420

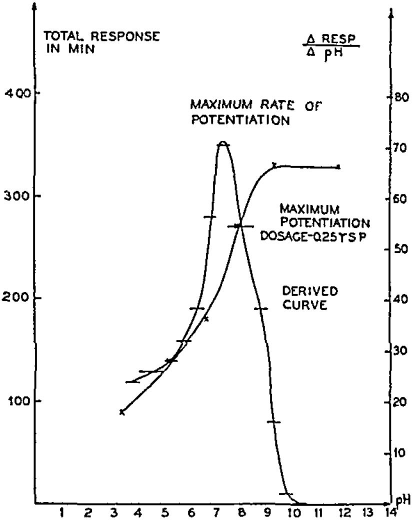

Fig 3

The above observations on the effect of pH on potentiation all point to the conclusion that the phenomenon of potentiation is one which involves a chemical change of the native hormone molecule The nature of the chemical transformation in potentiation will be discussed in the following section

2 *Potentiation and destruction of melanophore hormone by alkali* Two series of experiments using two media, $M/10\ Na_3PO_4$ and $N/1\ NaOH$, were conducted

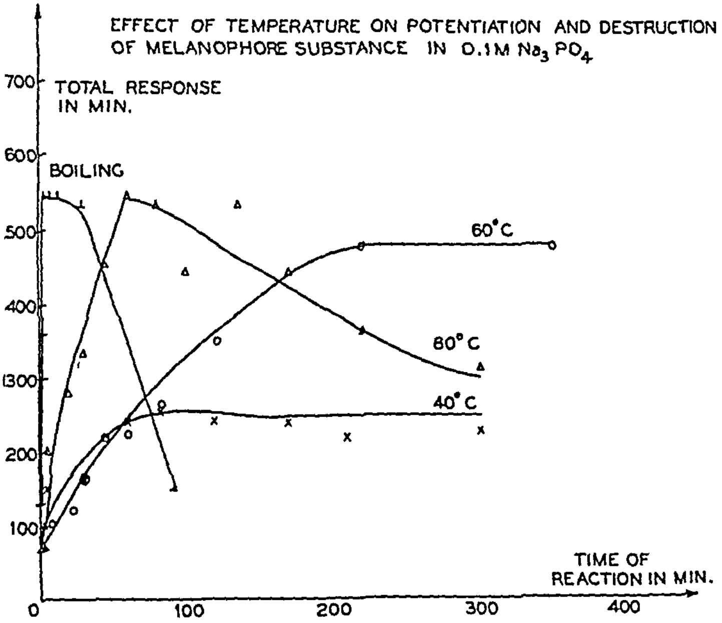
EFFECT OF TEMPERATURE ON POTENTIATION AND DESTRUCTION OF MELANOPHORE SUBSTANCE IN 0.1M Na_3PO_4
TOTAL RESPONSE IN MIN.
BOILING
60°C
80°C
40°C
TIME OF REACTION IN MIN.
700
600
500
400
300
200
100
0
100
200
300
400

Fig. 4

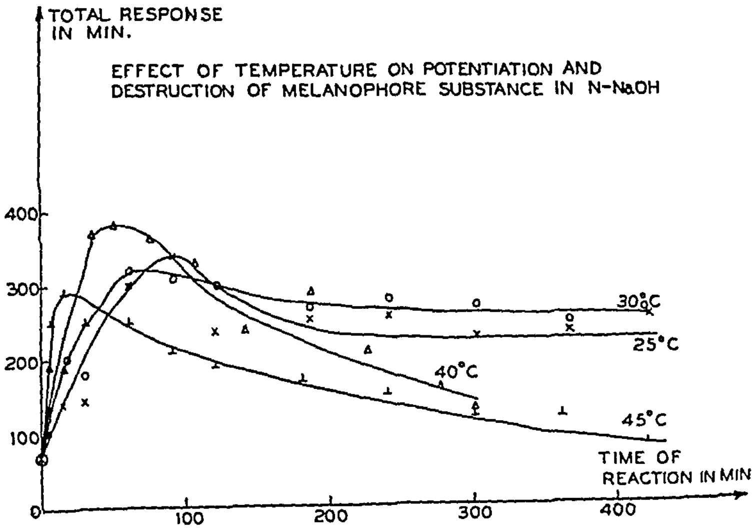
TOTAL RESPONSE IN MIN.
EFFECT OF TEMPERATURE ON POTENTIATION AND DESTRUCTION OF MELANOPHORE SUBSTANCE IN N-NaOH
30°C
25°C
40°C
45°C
TIME OF REACTION IN MIN
400
300
200
100
0
100
200
300
400

Fig. 5

at various temperatures. The data are graphically represented in figures 4 and 5. The striking features about them are: (a) the accelerated rate of potentiation with temperature, which is approximately doubled at every ten-degree increase, and (b) the increased destruction of the melanophore substance with the rise of temperature or alkalinity.

It may be seen in figure 4 that at temperatures below 60°C., potentiation in M/10 Na_3PO_4 is not complete even after prolonged heating. At boiling temperature, however, the reaction is completed in three minutes. M/10 Na_3PO_4 and three-minutes' boiling are the optimal conditions for complete potentiation of the melanophore hormone.

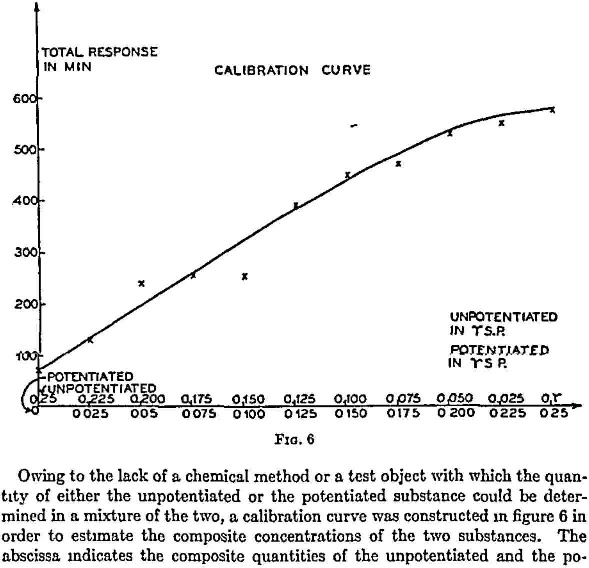

Fig. 6

Owing to the lack of a chemical method or a test object with which the quantity of either the unpotentiated or the potentiated substance could be determined in a mixture of the two, a calibration curve was constructed in figure 6 in order to estimate the composite concentrations of the two substances. The abscissa indicates the composite quantities of the unpotentiated and the potentiated substances, expressed in terms of standard powder, which is 0.25 γ in total. This is the equivalent amount injected into each frog. This range of composite concentrations is that of a potentiating medium in which the unpotentiated is changed into the potentiated substance. Using such composite solutions of the two substances, the total responses of frog melanophores were determined and plotted as ordinate on the graph. The standard solution of the posterior pituitary (made in 0.25% acetic acid) was used as the unpotentiated material. The potentiated material was made from the unpotentiated by boiling for three minutes in M/10 Na_3PO_4.

From the calibration curve, the total response on the potentiation graphs in figure 4 may be converted into the concentration of the unpotentiated material in the potentiation medium. By plotting the logarithm of concentration of the unpotentiated material in the potentiation medium against the time of the reaction, a linear relationship is obtained (fig. 7) for the potentiation graphs in figure 4. Such a relationship may be represented by the following equation:

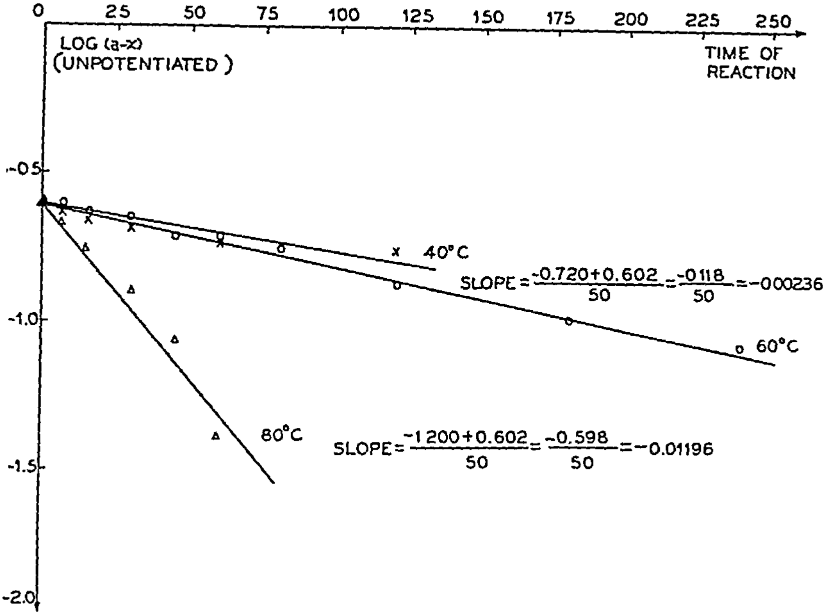

FIG. 7

$T = \frac{1}{K} \log \frac{a}{a - X}$, where a = concentration of the unpotentiated material at the beginning of the reaction, $T = 0$; X = amount of the material transformed during the time T; a-X = concentration of the unpotentiated substance at time T of the reaction; and K = velocity constant.

By differentiation, $\frac{dX}{dT} = K\,(a-X)$, which means that the reaction is of the first order, or a monomolecular reaction.

The velocity constant K for the potentiation reaction in M/10 Na_3PO_4 was found to be 0.0024 and 0.012 for 60°C. and 80°C. respectively.

Among the reactions which obey the law of the first order are those of molecular cleavage and rearrangement. The potentiation of melanophore hormone may be due to such an effect on the molecule.

Since there still remains the criticism that the concentration of the unpotentiated or the potentiated hormone was not estimated individually, the velocity of the potentiation reaction should be further investigated with a method, chemically or biologically specific for one of the two substances

As indicated in figure 5, the reaction is quantitatively different in N/1 NaOH Destruction of the hormone takes place slowly even at 25°C and increases rapidly with the rise of temperature It has not been possible to potentiate the melanophore hormone in N/1 NaOH by the regulation of temperature without at the same time encountering destruction It is evident that N/1 NaOH should not be used to potentiate melanophore hormone in quantitative studies

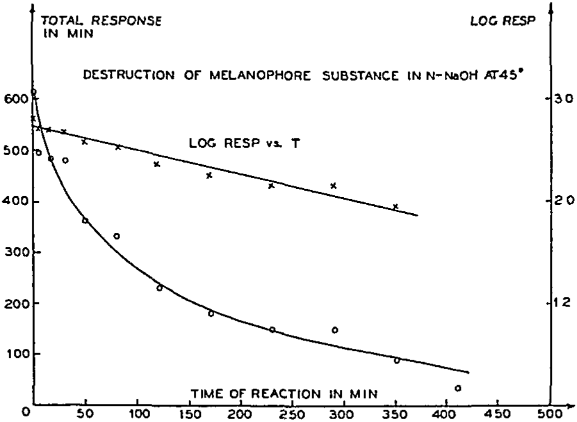

Fig 8

The graphs in figure 8 were obtained from an experiment in which the destruction of the potentiated hormone was conducted in N/1 NaOH at 45°C The potentiated material was first made in M/10 Na_3PO_4 and added to the reaction medium The straight line was obtained by plotting the logarithm of the total response as the ordinate and the time of the reaction in minutes as the abscissa It will be shown in a succeeding section that the logarithm of concentration and the logarithm of total response are related linearly, consequently, the logarithm of concentration and the reaction time are also linearly related Expressed mathematically the following equation holds

Let R = *total response*, X = *concentration of the potentiated substance at the reaction* T, since $R = aX^b$, $\log R = \log a + b \log X$, from the graph, $\log R =$

$A - KT$, therefore $\log a + b \log X = A - KT$, $\log X = \frac{A - \log a - KT}{b}$, $\log X = A' - K'T$ or $X = A'' e - K''T$ where $a, b, A, K, A', K', A'', K''$ are constants.

We have so far presented evidence indicating the chemical transformation of one melanophore substance into another by potentiation with alkali. In the following section, experimental evidence, which shows a difference in pharmacological properties between the potentiated and the unpotentiated material, will be given.

3. *The pharmacological properties of the unpotentiated and the potentiated melanophore substances.* In table 2 is given the amount of unpotentiated or potentiated material that will produce in 40–60% of the hypophysectomized frogs a maximal response as indicated by one of the following stages—semi-stellate, stellate, stellate-reticulate and reticulate. Each determination was made in 10 frogs, 7–10 days after operation. The unpotentiated and the potentiated materials were prepared according to the routine procedures as given in the above section.

TABLE 2

STAGE*	AMOUNTS REQUIRED IN γ OF STAND POWD.	
	Unpotentiated	Potentiated
S.S.	0.06 (0.02–0.1)	0.05
S.	0.18 (0.15–0.2)	0.08
S.R.	0.30 (0.25–0.35)	0.10
R.	0.60 (0.40–0.80)	0.15

* S.S., semi-stellate; S., stellate; S.R., stellate-reticulate; R., reticulate.

Arbitrarily giving a numerical value, 1, 2, 3, and 4, to indicate the relative increase in size of the melanophores to each of the successive stages—*SS*, *S*, *SR*, and *R*, and plotting against the logarithms of the doses required to produce the corresponding stages in 50% of animals, a linear relation is obtained as indicated in figure 9. The straight line for the unpotentiated substance has a different slope and a different intercept from that for the potentiated. It means that the reaction of the melanophores toward the two substances is qualitatively and quantitatively different.

A more striking picture of the melanophore-reaction towards these two substances may be seen from the time-response curves in figure 10. The stages of response were plotted against the time of the reaction. The readings of the various stages on the graphs were recorded from the observations of 10 frogs when 50% or more of the animals showed such reactions. The graphs represent the intensity and duration of response in hypophysectomized frogs after receiving an equivalent quantity of the unpotentiated or the potentiated melanophore material in terms of the standard powder. In the first place, they indicate that the activity of the melanophore substance is greatly increased by potentia-

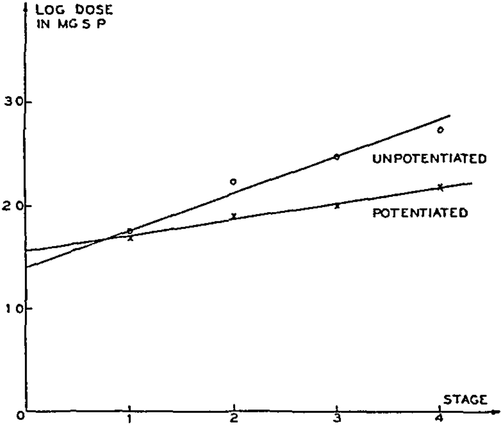
LOG DOSE
IN MG S P
3 0
2 0
1 0
0
UNPOTENTIATED
POTENTIATED
STAGE
1
2
3
4

Fig 9

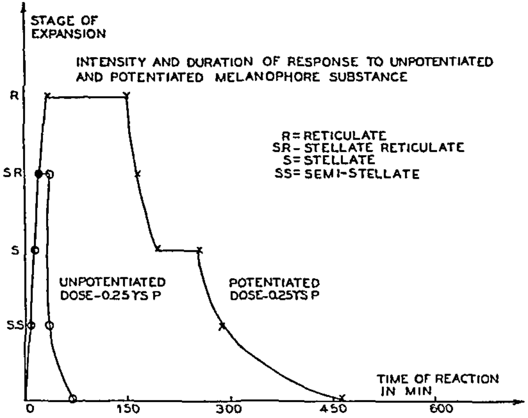
STAGE OF
EXPANSION
INTENSITY AND DURATION OF RESPONSE TO UNPOTENTIATED
AND POTENTIATED MELANOPHORE SUBSTANCE
R
SR
S
S.S
R = RETICULATE
SR - STELLATE RETICULATE
S = STELLATE
SS = SEMI-STELLATE
UNPOTENTIATED
DOSE-0.25 YS P
POTENTIATED
DOSE-0.25YS P
TIME OF REACTION
IN MIN
0
150
300
450
600

Fig 10

tion as shown by the increase of both the intensity and the duration of the melanophore response. Second, the rate of return to the contracted stage is much slower after injection of the potentiated than after injection of the unpotentiated material. This is especially so in the return from the stellate to the fully contracted condition.

These findings support our previous contention and the statement of Stehle that the unpotentiated and the potentiated materials are qualitatively and quantitatively different in their pharmacological effects (4). The potentiated substance, as Stehle believes, is probably of no physiological importance. The results are also in agreement with the recent observations of Landgrebe and Waring on Xenopus (5). The quantitative differences in the responses of unpotentiated and potentiated hormones have led us to make an extensive investigation of the relation between dosage and total response of the two substances. This will be discussed in detail in the following section.

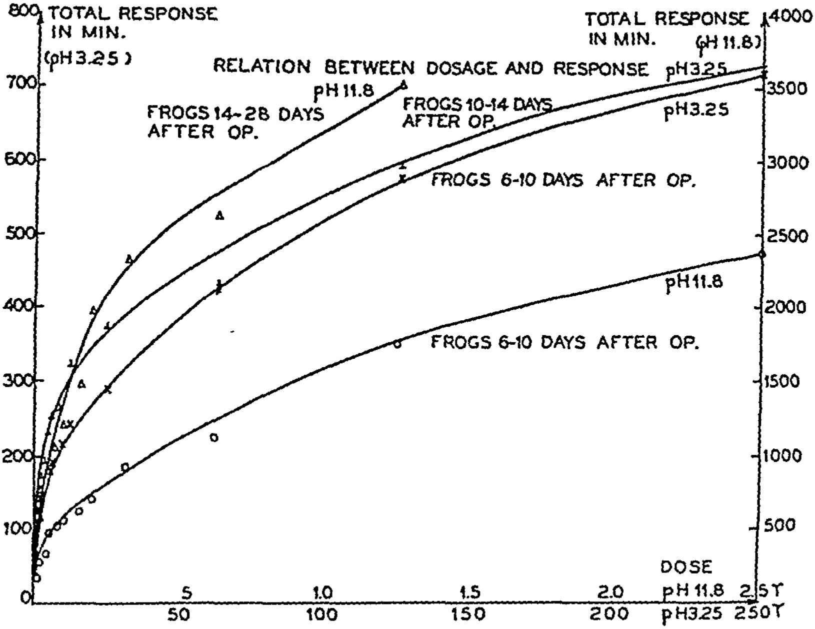

FIG. 11

4. *The relation between dose and the duration of response.* When the data, graphically presented in figure 11, are plotted as their logarithms, as in figure 12, there is obtained a linear relationship. This relation may be expressed by the following equation:

Since $\log_{10} R = a + b \log_{10} X$ (R = total response, X = dose, a and b constants—the intercept and the slope of the straight line respectively) $\log_{10} R/X^b = a$, $R = 10^a X^b$.

Some important characteristics may be pointed out from the lines in figure 12 In the first place, the linear relationship between the logarithm of dose and that of response holds true for the unpotentiated as well as for the potentiated substance The difference lies in the two constants *a* and *b* From the numerical values calculated from the lines in figure 12, it is of interest to note that the slope of the lines is the same for each substance regardless of the sensitivity of the test frogs, the degree of sensitivity being dependent upon the time interval following hypophysectomy The value of *b*, the slope of the line, was found to be approximately $\frac{1}{2}$ for the potentiated material and $\frac{1}{3}$ for the unpotentiated The value *a*, on the other hand, varies accordingly with the sensitivity of the animal—the longer the time following hypophysectomy, the greater the value for *a* In other words, the value of *b* is dependent upon the chemical nature of the melanophore substance, while the value of *a* is determined by the sensitivity of the test animal

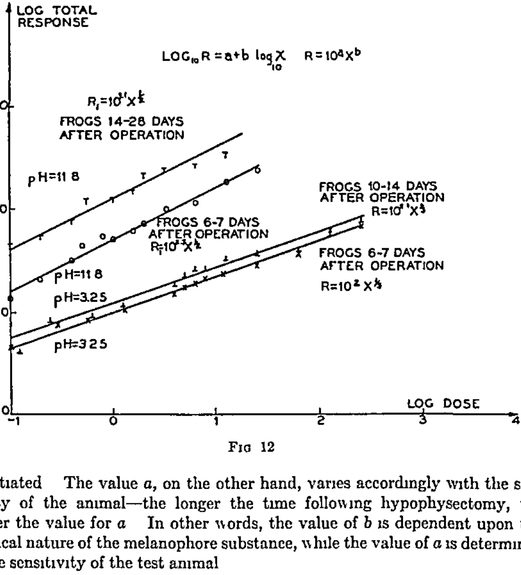

FIG 12

It may be mentioned also that the equation as given above is the well known one for adsorption isotherm A discussion of the physico chemical laws in cell drug reactions is beyond the scope of the present paper. However, the reader is referred to Clark's text for a discussion of this topic (6, 7)

The relation between dose and total response has been described in order to interpret the data obtained for the destruction of the melanophore hormone By the same relationship between dose and response for the potentiated and unpotentiated substances, a numerical expression may be formulated for potentiation. Based upon the total response as a measure for the activity of the melanophore substance, potentiation may be defined as the ratio of the total response of the potentiated to that of the unpotentiated substance. It follows then:

$$P\ (Potentiation)\ \frac{R_1}{R} = \frac{10^{a_1} X^{b_1}}{10^{a} X^{b}} = 10^{a_1 - a} X^{b_1 - b}$$

Taking the two equations, $R_1 = 10^{2.7} X^{1/2}$ and $R = 10^{2} X^{1/3}$ for the potentiated and unpotentiated substances, calculated from the results of two experiments which were conducted on the same day with frogs of similar sensitivity (6–7 days after hypophysectomy), the equation of potentiation becomes:

$$P = 10^{2.7-2} X^{1/2-1/3} = 5X^{1/6}.$$

The values for potentiation calculated from this equation and those obtained from the observed data are given in table 3. It is evident that taking the duration of response as a measure of the activity of the potentiated and that of the unpotentiated substances, it is not possible to state with a single value the degree of potentiation.

TABLE 3

DOSAGE IN γ S.P. x	DURATION OF RESPONSE		POTENTIATION T_1/T*	
	pH 3.25 (T)	pH 11.8 (T_1)	Obs.	Calc.
	min.	*min.*		
0.1	45	135	3.0	3.4
0.4	75	315	4.2	4.3
1.2	108	540	5.0	4.9
6.3	190	1115	5.8	6.8
12.5	240	1800	7.5	7.3
25.0	290	2380	8.2	8.6

* $\frac{T_1}{T} = 5X^{1/6}$ $\log \frac{T_1}{T} = 0.699 + 1/6 \log x.$

Another way of expressing potentiation is in terms of the ratio of concentration of the unpotentiated melanophore substance to that of the potentiated, which will produce the same duration of response of the melanophores. Theoretically, it may be shown from the dose-response relationship that the quantities of the unpotentiated and the potentiated substances which would give the same duration of response are related as follows: when $R_1 = R$, $X_1^{b_1} = X^{b} 10^{a-a_1}$ where X_1 and X are quantities or concentrations of the potentiated and the unpotentiated substances respectively in terms of the standard powder.

Following this definition, the values of potentiation in table 4 were calculated (*a*) from the data on the graphs in figure 11 and (*b*) by the linear relationship between the logarithm of dosage and that of response. It may be seen that the value of potentiation is greater the longer the duration of response chosen for comparison. In other words, this value varies also with the dosage.

An application of the linear relation between the logarithm of dose and that of total response is in the estimation of melanophore substances. This will be discussed in the succeeding section on the methods of assay of melanophore material.

5. *Methods of assay.* The activity of a melanophore substance has been determined in one of the following two ways: (*a*) by the intensity and (*b*) by the duration of response of melanophores in test animals (1, 8, 9). Regardless of the test objects or how the substance is to be assayed, it is clear from the observations presented in this paper that it is of paramount importance to state the pH and the temperature employed in the preparation of the melanophore substance. Otherwise the estimated value will have no quantitative significance, since it may be a measure of one or a mixture of the unpotentiated and potentiated melanophore substances, the quantities of which vary with the pH and the temperature employed. Since no method is available which would determine either one of the two melanophore substances, one way of estimating the activity of an unknown melanophore sample is by comparing it with a standard prepared at the same pH and temperature. Obviously, one meets considerable difficulty in preparing a standard under conditions identical with those used for the unknown, without encountering some loss of the melanophore activity. In order to avoid the experimental difficulties, the simplest procedure is to determine the melanophore activity in either one of the two forms, the unpotentiated or the potentiated substance. In case of a mixture of the two the material may be estimated in the potentiated condition by first converting the unpotentiated into the potentiated substance. This is the criterion adopted in the assay methods which will be described.

TABLE 4

TOTAL RESP.	POTENTIATION* VALUE CALCULATED	
	From graphs	From formulae
minutes		
200	43	50
300	92	77
400	110	100
500	119	126
600	121	152
700	128	178

$$* \text{ Potentiation} = \frac{\text{amt. of unpot. melanophore substance}}{\text{amt. of pot. melanophore substance}}.$$

The logarithmic relation between doses and the various stages of response, as indicated by the graphs in figure 9, offers an intensity method for the estimation of the unpotentiated or the potentiated melanophore substance. By means of such a calibration line, the activity of the unknown may be calculated from the stage of response which occurs in 50% of the 10–20 frogs. Instead of securing a 50% response, it is simpler to record the individual responses and calculate the activity from the average value of the stages in all animals, using the numerical values arbitrarily chosen for the various stages.

With the intensity of response as an index of the activity of the melanophore substance, a unit of the melanophore hormone may be defined as *that amount of standard powder which, when injected into each of twenty hypophysectomized frogs weighing 25–30 grams one week after operation, will produce a reticulate response of the melanophores in 50% of the animals.* Naturally, the unit is different for the two melanophore substances; it was found to be 0.5–0.6 γ of the standard powder for the unpotentiated and 0.15 γ of the standard powder for the potentiated melanophore substance. By this method of assay then, the potentiated material is about four times as potent as the unpotentiated when the degree of potentiation is expressed by the inverse ratio of the quantities of two substances in terms of the standard powder that would be required to produce the same intensity of response of the melanophores. This expression gives an absolute value of potentiation.

This method would be both simple and accurate if the various stages of response could be distinctly read and the area of the melanophores could be quantitatively recorded at the proper times. Such objective variations plus a subjective factor limit the method to the use of a small number of animals, and that inherently affects its accuracy. Furthermore, to be quantitative, a calibration curve should be constructed from a standard every time. This is also a laborious procedure.

Evidently, the intensity method cannot be used for an amount of the melanophore substance which produces a reticulate response in all the animals.

Owing to its simplicity, accuracy and applicability over a wide range of concentrations, we prefer the following method of assay which is based on the duration of response as an index for the activity of a melanophore substance.

The principle of the method rests upon the linear relation between the logarithm of the dose and the logarithm of the time of total response, which has been described in previous sections. For practical reasons, it has been found desirable to limit the range of doses to such quantities as will give a total response time from one to ten hours. The individual variation and the sudden augmentation of the duration of response to an increasing quantity of melanophore substance at small dose levels render the determinations inconsistent with very small doses. Therefore, it is best to begin with a dose that will produce at least an hour's response.

The procedure we used was as follows: First, an approximate estimation of the activity of an unknown melanophore sample was secured. For this, two pairs of frogs were injected with the unknown solution in two different concentrations, and watched for the intensity and the duration of response. Then, in order to make a quantitative determination, groups of four frogs, approximately of the same weight, were selected and injected with a standard and with an unknown solution. Four to six concentrations of a standard and two concentrations of an unknown solution, within the range of concentrations of the former, were used. By the duration of total response, the concentration of the unknown solution was calculated from the log-log straight line relation between dose and total response of the standard solution. A statistical study of the assay method will be made later.

For convenience in the calculation of the activity of a melanophore preparation, a unit of melanophore activity may be adopted. 0.5 γ of the standard powder appears to be an appropriate quantity for the unpotentiated substance; it will give a reticulate response in 50% of hypophysectomized frogs and a duration of response of two hours. For the same reason, 0.15 γ of standard powder would be a suitable quantity for the potentiated substance; this will give a total response of eight hours in frogs one week after hypophysectomy.

SUMMARY

The melanophore hormone may be completely potentiated at pH 11 after three minutes' boiling or at pH 9 after two hours. Owing to an equilibrium condition established between the unpotentiated and the potentiated substance at a pH lower than 9, potentiation does not undergo completion even after prolonged boiling.

The reaction of potentiation is of the first order, the increasing rate of potentiation is approximately doubled at every ten-degree increase of temperature.

Destruction of the melanophore hormone occurs slowly in N/1 NaOH at 25°C., and increases rapidly with the rise of temperature or alkalinity.

The unpotentiated and the potentiated melanophore substances are qualitatively and quantitatively different in their pharmacological effects as is indicated by the intensity and the duration of response of the melanophores.

The intensty of the melanophore response may be used as an index for measuring the activity of a melanophore preparation. This is based upon a logarithmic relation between dosage and the extent of expansion of the melanophores

When the logarithms of the time of the total response are plotted against the logarithms of dosage, a straight line is obtained for both the unpotentiated and the potentiated melanophore substances. However, the slope and the intercept of the straight line for the unpotentiated substance are different from those for the potentiated. This linear relationship between the logarithm of dosage and that of the duration of response is utilized for the quantitative assay of the melanophore activity of the unpotentiated and the potentiated preparations.

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STUDIES ON VERATRUM ALKALOIDS

III. Qualitative and Quantitative Differences in the Action of Cevine and Veratridine[1]

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Veratrine manufactured from the seeds of *Veratrum Sabadilla*, Aiton, or *Schoenocaulon officinale*, Gray, is a mixture of various alkaloids. Its chief constituents are cevadine, veratridine, and cevine. Cevadine is the tiglic acid ester of cevine, and veratridine is the veratric acid ester of the same base. Previous studies made with the pure alkaloids have dealt essentially with the quantitative relation of the three alkaloids, while qualitative differences either have not been observed or have not been sufficiently emphasized. Cevadine and an amorphous alkaloid which was probably veratridine were first compared by Lissauer (1) in 1877, who found no differences between the two alkaloids. In 1899 Falk (2) administered cevine and cevadine to frogs and rabbits, and the brief note referring to his studies suggests some qualitative difference between these two substances.

The present investigation was undertaken to extend our knowledge of the action of veratridine and cevine and of the quantitative relation between their effects, and to ascertain the existence of qualitative differences between the two substances. Certain observations on veratrine are included for comparison. Unless otherwise indicated, all dosages mentioned throughout this paper in connection with cevine, veratrine, and veratridine refer to cevine hydrochloride + $2H_2O$, veratrine hydrochloride, and veratridine base respectively. The molecular weights of cevine and cevine hydrochloride dihydrate are 509 and 581 respectively, and that of veratridine is 673.

The veratridine and one of the samples of cevine used in our experiments were isolated by Professor R. P. Linstead and Dr. D. Todd from veratrine hydrochloride Merck (3). The other sample of cevine was supplied to us by Dr. Walter A. Jacobs. There was no difference in the biological action of the two samples of cevine.

Methods. *Isolated frog heart:* The experiments were carried out on *Rana pipiens*. The hearts were isolated according to Straub or were perfused from the venous side using the technique described by Bulbring (4). The physiological salt solution had the following composition (Clark): NaCl 0.65%; KCl 0.014%; $CaCl_2$ 0.011%; $NaHCO_3$ 0.02%.

Comparative toxicity in unanesthetized mammals: White rats weighing from 100 to 150 grams were used. The minimum lethal dose and mortality curve were determined by injecting each dose intraperitoneally to groups of twelve animals and plotting dose against percentage mortality according to the method of Behrens (5).

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Anesthetized and spinal cats. The animals were anesthetized by the intraperitoneal administration of 35 to 40 mgm. of nembutal per kgm. body weight. Respiration was recorded by means of a Marey tambour connected to a tracheal cannula. Spinal cats were prepared under either anesthesia by cutting the spinal cord at the level of the second cervical vertebra and destroying the brain by a probe thrust through the *foramen magnum*. Cats used to study the liberation of epinephrine from the adrenal glands were anesthetized with nembutal and given artificial respiration. One eye was enucleated to facilitate the recording of the nictitating membrane. The membrane was denervated by removing the corresponding superior cervical ganglion at least 12 days previous to the day of the experiment. The vagi were cut to avoid vagal reflex action and the aorta and inferior vena cava were ligated below the renal arteries after evisceration. A cannula was placed into the celiac artery and injections were made into the aorta so that the drugs reached the adrenals little diluted with blood. Blood pressure was recorded by means of a mercury manometer from the abdominal aorta just above the iliac arteries.

RESULTS. 1. *Experiments on the normal frog and on the isolated frog heart.* In normal frogs 0.01 mgm. of veratridine per gram body weight injected into the ventral lymph sac causes muscular and respiratory paralysis in a few minutes, accompanied by gasping movements and fibrillary twitchings of the hind legs. This is followed by some spontaneous movements of the legs and by fits of tetanic convulsions of very short duration. The convulsions disappear only after the destruction of the spinal cord. Death occurs approximately one hour after the injection. The heart stops in systole.

Cevine in a dose of 0.1 mgm. per gram body weight does not lead to convulsions. 0.5 mgm. per gram body weight causes an effect similar to that previously described for 0.01 mgm. of veratridine with the difference that the convulsive effect which appears after a preliminary period of paralysis is more pronounced. A dose of 1 mgm. per gram body weight of cevine does not cause systolic standstill after 5 hours, instead auriculo-ventricular block accompanied by a very slow heart rate occurs.

In the frog heart isolated according to Straub, veratridine 1×10^{-6} or cevine 1×10^{-4} causes a decrease in heart rate with an increase in the amplitude of contraction. Ventricular extrasystoles and auriculo-ventricular block are sometimes observed with these concentrations. A concentration of 1×10^{-5} of veratridine or 2×10^{-3} of cevine causes an immediate "systolic effect." The height of the systole increases and the relaxation of the ventricle is not complete. Auriculo-ventricular block and extra-systoles with periodic stoppages of the heart in "half systole" appear during the first few minutes of the action. This is followed by a very pronounced lengthening of the ventricular systole, which lasts from 1.5 to 2.5 seconds. At this stage the block does not seem to be due to an impairment of A-V conduction alone, but also to the fact that some auricular contractions occur while the ventricle is still contracted. Systolic standstill is brought about by a concentration of 2×10^{-5} (3×10^{-5} M) of veratridine and 1×10^{-2} (1.7×10^{-2} M) of cevine (figure 1). The relative activity of veratridine and cevine is therefore approximately 1 to 500. The effects of cevine and veratridine on the frog heart are reversible by washing.

No qualitative differences can be discovered in the action of veratridine and cevine on the isolated frog heart by using the Straub technique. Their effect is apparently analogous to that of veratrine. The action of veratrine on the frog heart has been studied by Seeman and Victoroff (6) and de Boer (7), and that of cevadine by Lissauer (1) and Boehm (8). Their qualitative findings are similar to ours with veratridine and cevine.

The hypodynamic frog heart was used to ascertain the "therapeutic" potency of cevine, veratridine, and veratrine. In this series the hearts were perfused from the venous side, given a constant venous supply, and the output measured. A hypodynamic state was induced by perfusion with a Clark solution containing only 25 to 50% of the normal content of calcium. Perfusion with the drug dissolved in calcium-poor Clark solution started when the output was reduced to 50% or less of the initial value. Each heart preparation was treated with only one concentration of a single alkaloid. A representative experiment is given in table 1. In this particular experiment cevine was used in a concentration 2×10^{-4}. This concentration of cevine rapidly restored to nearly its normal value the decreased output and the stroke volume of the heart. In another experiment in which a concentration of 1.66×10^{-4} was used an analogous result was obtained. A concentration of 1×10^{-4} proved to be ineffective in two experiments. In these four experiments the hypodynamic state was induced with Clark solution containing 50% of its normal content in calcium.

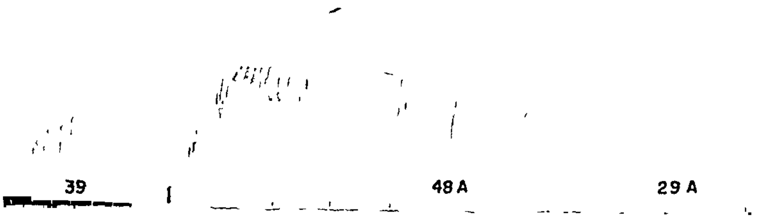

FIG. 1. The effect of cevine on the isolated frog heart (Straub technique). At sign, Clark solution was replaced by cevine 1×10^{-2}. Figures indicate heart rate per minute; A means auricular rate. Time in 10-second intervals. In the second half of the curve, four incomplete ventricle diastoles occurred.

The concentrations of veratridine (12 experiments) and of veratrine (7 experiments) which would restore normal output were found to be between 5×10^{-7} and 1×10^{-7} for veratridine and between 5×10^{-7} and 2.5×10^{-7} for veratrine. Because of the variability of our results, more experiments than we were able to make because of the limited supply of the pure alkaloids would be necessary for an accurate comparison of veratridine and cevine.

2. *Comparative toxicity of veratrine, veratridine, and cevine in rats.* Lethal doses of veratrine and veratridine produce a similar and rather characteristic picture of poisoning. The first signs generally appear within 10 minutes after intraperitoneal administration. They consist in salivation and a peculiar jerking movement of the head which repeats itself at short intervals. The respiration becomes very slow and gasping, cyanosis appears, the heart rate becomes slow

and irregular, and the animal sits or lies down but occasionally suddenly erects itself and stands on its hind legs for several seconds Jerking of the whole body accompanied by small jumps is sometimes observed The striated muscles are stiff and walking movements are performed with great difficulty Death usually occurs within $1\frac{1}{2}$ hours after injection

Lethal poisoning by cevine produces the following picture, which in some respects differs essentially from that presented by veratrine and veratridine Profuse salivation and a fine continuous tremor appear within 5 minutes after administration This is followed by violent jerks and by a very pronounced increase in reflex excitability which makes the animal jerk upon mechanical or auditory stimulation The tremor and jerking are most pronounced about 30 minutes after injection Soon afterward the animal lies down on its belly with its legs and tail in extension This is followed by occasional seizures of tonic clonic convulsions Death occurs within 2 to 4 hours, usually during an attack of convulsions In the animals receiving a sublethal dose the tremor lasts for several hours Falk (2), in his short account of the action of cevine, mentioned its convulsive action in the rabbit

TABLF 1

The action of cevine on the hypodynamic frog heart

TIME	OUTPUT	HEART RATE PER MIN	STROKE VOLUME
minutes	*cc/min*		*cc*
0	5 0	46	0 108
3	5 0	44	0 113
5	5 2	48	0 108
6	Clark solution containing 50% of its normal content of $CaCl_2$		
8	3 8	46	0 082
11	3 3	50	0 066
13	2 6	44	0 059
15	2 0	14	0 045
16	Cevine 2×10^{-4} in Clark solution containing 50% of its normal content of $CaCl_2$		
18	2 2	44	0 050
20	3 1	46	0 067
22	4 1	44	0 093
24	4 7	46	0 102
26	4 9	46	0 106
28	4 7	46	0 102
30	4 6	46	0 100
32	4 5	46	0 098

The mortality curve of the three substances when administered by intraperitoneal injection is given in figure 2 Their minimum lethal dose (L D 50) in milligrams per 100 grams body weight are as follows cevine 7 7, veratrine 0 48, veratridine 0 35 The relative toxicity of the three substances is, therefore, cevine 1, veratrine 16, and veratridine 22 (on a molar basis, cevine 1, veratridine 25)

3 *Circulatory effects of cevine and of veratridine* The cardiac effects of cevine,

veratridine, and veratrine on the isolated mammalian heart have recently been studied by Krayer and Mendez (9) and by Moe and Krayer (10). The paper by Moe and Krayer is the first in which the circulatory effect of cevine is mentioned, but these experiments on the mammalian heart reveal only the quantitative differences between the activity of veratridine and cevine.

Von Bezold and Hirt (11) discovered in 1867 that the "alkaloid" veratrine caused a cardiac slowing and a fall in blood pressure of a reflex nature, and this was confirmed by Jarisch and Richter (12) using veratrine. Jarisch and Richter also found that after repeated doses had been administered, and especially when the vagi were blocked by cooling, an injection of a large dose of veratrine frequently resulted in an increase in blood pressure accompanied by an acceleration in heart rate.

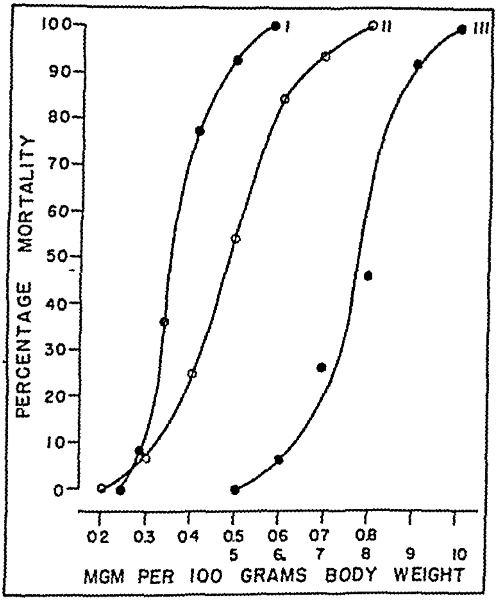

Fig. 2. Mortality curves of veratridine (I), veratrine (II), and cevine (III) on rats weighing 100 to 150 grams. The lower row of numbers on the abscissa expresses mgm. per 100 grams body weight for curve III (cevine).

A dose of veratridine of 0.1 mgm. (figure 3B) causes an effect on the blood pressure and respiration of a cat under nembutal anesthesia similar to that of a somewhat higher dose of veratrine. It consists of a fall in blood pressure accompanied by a decrease in heart rate and a sharp and transient stoppage of the respiration. The duration of the effect depends on the dose administered. In our experiments the effect of a dose of 0.1 mgm. of veratridine or of 0.2 to 0.3 mgm. of veratrine lasted from 20 to 40 minutes.

The effect of cevine on the blood pressure differs essentially from that of veratrine or veratridine. A dose of 20 to 30 mgm of cevine (figure 3A) causes no other circulatory change than a small increase in blood pressure with no appreciable change in heart rate. Doses of this magnitude or greater, depending on the depth of anesthesia, are apt to produce convulsions which mask further possible circulatory changes. These results reveal a qualitative difference between cevine and veratridine.

Repeated intravenous injections of veratrine or veratridine in a vagotomized cat cause an increase in blood pressure accompanied by an increase in heart rate The phenomenon is particularly well obtained in the spinal cat (figure 4). Using this preparation the increase in blood pressure is usually obtained with the first dose, but in one experiment out of four the first injection caused a momentary increase followed by a decrease The characteristics of the response suggested the possibility that it could be mediated by a liberation of epinephrine from the suprarenal glands and, as figure 4 shows, the increase in blood pressure obtained in the spinal cat with a dose of 0 3 mgm of veratrine is greatly reduced after ligation of the suprarenals. The small elevation occurring after the ligation of the adrenals might well be due to the effect of the second dose of veratrine upon the striated muscles

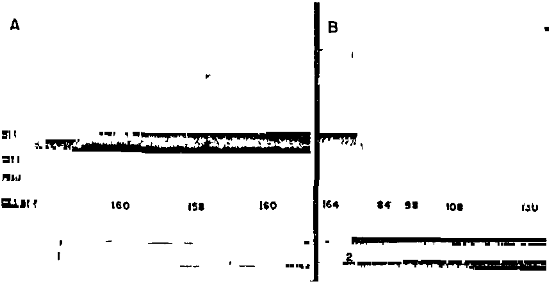

Fig 3 Effect of
2 53 kgm Anesth

intervals

Further and more direct evidence of the rôle of the suprarenal glands in the elevation of blood pressure was obtained by injecting the veratrine or veratridine into the celiac artery of a vagotomized and eviscerated cat, using the increase in blood pressure and the contraction of the nictitating membrane as indicators of the release of epinephrine from the adrenal glands Figure 5 shows the results recorded in an experiment of this type In this particular case vera-

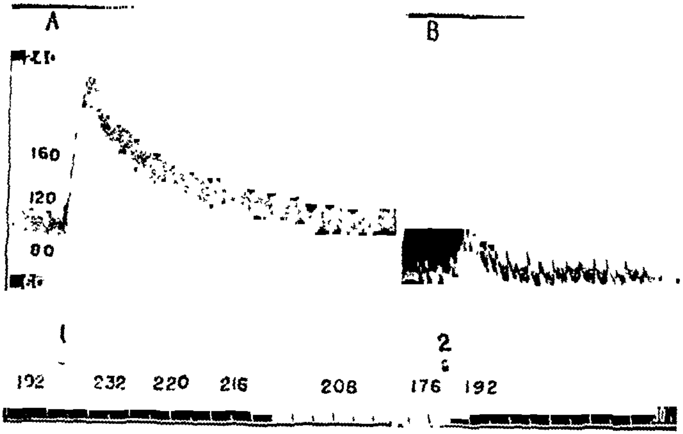

Fig. 4. Effect of veratrine on the blood pressure of the spinal cat. Cat weight 2.52 kgm. At *1* and *2*, 0.3 mgm. veratrine into the femoral vein. Adrenals were ligated between A and B. Row of numbers indicates heart rate per minute. Scale on left indicates arterial blood pressure in mm. of mercury. Time in 30-second intervals.

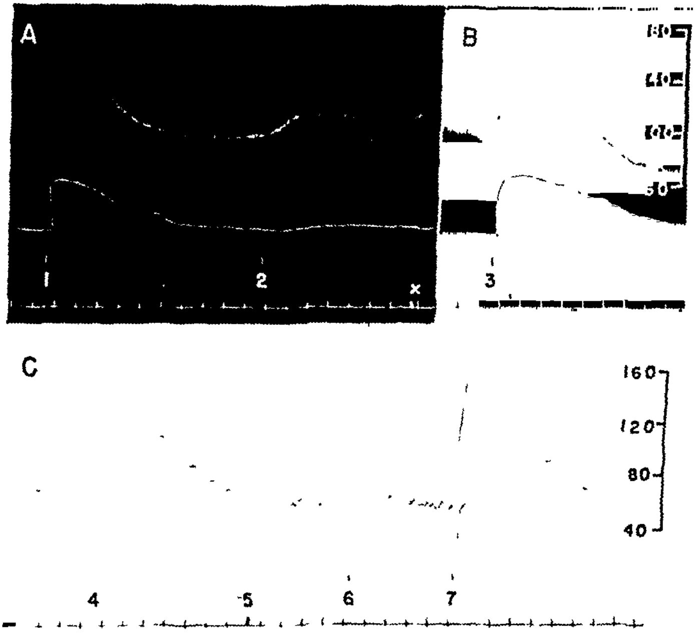

Fig. 5. Effect of veratridine on the release of epinephrine from the adrenal glands. May 26, 1942. Cat weight 2.35 kgm. Right superior cervical ganglion removed on May 14, 1942. Anesthesia, nembutal, 40 mgm. intraperitoneally. Vagi cut. Animal eviscerated. Tracings from top to bottom: arterial blood pressure (scale on right in mm. of mercury); nictitating membrane, signal magnet, time in 1-minute intervals. *1*, epinephrine hydrochloride 0.012 mgm.; *2*, cevine 5 mgm.; *3*, veratridine 0.2 mgm.; *4*, epinephrine hydrochloride 0.018 mgm.; *5*, adrenals ligated; *6*, veratridine 0.2 mgm.; *7*, epinephrine hydrochloride 0.018 mgm. Epinephrine hydrochloride was injected into the right jugular vein. Cevine and veratridine were injected into the celiac artery. Between A and B, 15 minutes. C, continuation of B. At X the drum was arrested for 4 minutes.

TABLE 2

The action of cevine on a rabbit under nembutal anesthesia. January 21, 1943. Female rabbit. 1.9 kgm.

TIME	RESP. RATE PER MIN.	HEART RATE PER MIN.	REACTION TO PAIN	EYELID REFLEX	CORNEAL REFLEX	TEMP.	REMARKS
						°C	
10:05	68	252	++++	++++	++++	38.8	
10:15	66	244	++++	++++	++++	38.8	
10:20	Nembutal intraperitoneally 30 mgm./kg.						
10:35	30	240	+++	++	++	38.3	Lying on its side. Eyeball movements present
10:45	25	232	+++	++	++	38.0	
10:55	24–26	224	+++	++	++	37.8	Same as above
11:05	26	220	+++	++	++	37.5	Same as above
11:07	Cevine 10 mgm. intravenously						
11:09	30	232	+++	+++	+++		Gnawing movements
11:12	30	224	+++	+++	+++	37.4	Gnawing movements. Hiccough
11:15	29	224	+++	+++	+++	37.4	
11:20	30	220	+++	+++	+++	37.4	Gnawing movements Hiccough
11:25	30	220	+++	+++	+++	37.3	Less gnawing movements
11:30	29	220	+++	+++	+++	37.3	
11:35	30	220	+++	+++	+++	37.2	
11:38	Cevine 10 mgm. intravenously						
11:39	28						
11:41	29	224	+++	+++	+++		
11:43	30	224	+++	+++	+++	37.2	
11:50	31	224	++++	++++	++++	37.1	Tries to raise head, moves eyeball, and make gnawing movements
11:55	31	224	++++	++++	++++	37.1	
12:00	33	220	++++	++++	++++	37.0	
12:05	36	220	++++	++++	++++		
12:12	Cevine 10 mgm. intravenously						
12:13	35						
12:14	34	232	++++	++++	++++		
12:16	32	232	++++	++++	++++	37.0	Increased reflex excitability
12:21	40	232	++++	++++	++++		Raises head. Stays on its belly with improved posture
12:34	44	236				37.0	
12:44	44	236	++++	++++	++++	37.1	Convulsive movements of the extremities
12:55	54	232	++++	++++	++++		Fine tremor
1.05	56	240	++++	++++	++++	37.2	Head erect. Greatly increased reflex excitability
1:25	52	260	++++	++++	++++	37.4	Generalized tremor
1:35	43	268	++++	++++	++++	37.4	Tries to get in sitting position moving the forelegs. Does not move hind legs
1:45	48	268	++++	++++	++++	38.0	Forelegs in extension. Generalized tremor
2:00	48	268				38.5	Tremors not so continuous

tridine was used. In other experiments veratrine was injected and the same results were obtained. A dose of 0.2 mgm. (figure 5) of veratridine caused an increase in blood pressure and a contraction of the nictitating membrane approximately equal to changes produced by 0.018 mgm. epinephrine hydrochloride when injected into the jugular vein. In the same experiment cevine was injected in a dose of 5 mgm. This caused an increase in blood pressure less pronounced and much longer lasting than that caused by veratridine and had no effect on the nictitating membrane. The same results were obtained in in another experiment in which 10 mgm. of cevine were injected. This dose could not be surpassed as the convulsive action of the drug interfered with the proper recordings.

4. *The respiratory effect of veratridine and cevine and the convulsant action of cevine.* Veratridine has the same qualitative effect as veratrine on the respiration. In the cat a small dose of veratrine or veratridine causes a transient stoppage of the respiration (figure 3). Bigger doses may kill the animal by respiratory failure. Figure 3 also shows that in the cat a sub-convulsive dose of cevine causes a small and evanescent decrease in respiratory rate followed by a small increase. Bigger doses of cevine do not further decrease the respiratory rate, but, on the contrary, increase it as a result of a stimulating central action that leads to convulsions. Between part A and part B of figure 3 a second dose of cevine (20 mgm.) was injected. This additional dose led to clonic convulsions that were abolished by an intravenous injection of 20 mgm. of nembutal. This and two other experiments in cats in which anesthesia was induced by nembutal showed the convulsant dose of cevine to be dependent upon the dose of nembutal previously given or upon the depth of the general anesthesia.

This antagonism between nembutal and cevine suggested that cevine might be regarded as a convulsant substance possessing analeptic properties. To test this assumption three experiments were made in rabbits under nembutal anesthesia, and table 2 illustrates as an example the uniform results obtained.

It is obvious from table 2 that cevine has analeptic properties but that its analeptic effect is not likely to be of practical value. The drug does not seem to exert any specific effect on the respiratory center as the increase in respiration is not apparent until signs of convulsive activity are noticeable. The same is true of the improvement of postural reflexes and of the state of wakefulness.

Discussion. The results of the frog heart show that if the toxic activity of cevine is considered 1, that of veratridine is approximately 500 on a molar basis. Moe and Krayer (10) found that the therapeutic potency of cevine is less than 1/300 of that of veratridine in the isolated mammalian heart.

As we have already pointed out, no qualitative difference between the effect of cevine and that of veratridine on the isolated heart could be observed. In view of the recent assumption by Craig and Jacobs (13) that the veratrum alkaloids may be derivatives of sterol and therefore more closely related to the cardiac aglycones than was thought heretofore, it is of interest to emphasize that cevine and its esters share with the cardiac glycosides and aglycones the property of bringing about systolic standstill of the frog heart and the ability to restore the normal activity of the isolated hypodynamic frog heart and of the failing isolated mammalian heart.

A distinct qualitative difference between cevine and veratridine is shown in their effect on mammals. While cevine, which has a primary convulsant action causes an increase in respiratory rate, veratridine acts as a powerful respiratory depressant, causing the death of the animal by respiratory paralysis. The rat mortality curves show that if the toxicity of cevine in the rat on a molar basis is considered as 1, that of veratridine is of the order of 25. There is, therefore, a marked discrepancy between the relative action of cevine and veratridine upon the isolated mammalian heart and the relative toxicity of these two substances in the normal mammal. The explanation of this discrepancy can probably be found in the convulsant properties of cevine. Although veratridine shows convulsant activity in the frog, it cannot exhibit it in the normal mammal because of its powerful primary effects on the respiration and on the vagal control of the heart. Some antogonism between cevine and barbiturates results from the central stimulation by cevine, but our experiments suggest that the analeptic property of the drug cannot be compared with that of well known analeptics such as metrazol and picrotoxin.

The action of veratridine on the blood pressure and respiration is indistinguishable from that of veratrine. Marked qualitative differences, however, are observed if cevine is compared with veratridine. A dose of 20 mgm. of cevine, i.e., a dose 200 times greater than the effective dose of veratridine, causes no fall in blood pressure but a small rise, probably due to its nervous or muscular action. This suggests that cevine has comparatively little or no action on the reflex vagal control of the circulation.

The release of epinephrine from the suprarenal glands is probably the cause of the increase in blood pressure and in heart rate produced by veratridine and veratrine in the spinal cat and one of the factors involved when the same phenomenon is observed after repeated injections in the vagotomized animal. According to Richter and Thoma (14) another factor might be a direct stimulation of the vasomotor center. While it is not possible to say from our experiments whether doses of cevine larger than those employed by us will have an action upon the suprarenals, veratridine on a molar basis is at least 70 times as potent as cevine in its ability to increase the liberation of epinephrine.

The release of epinephrine from the suprarenal glands caused by veratrine and veratridine has not been adequately considered in the past, although Kusnetzow (15) showed an increase in the liberation of epinephrine from the isolated suprarenal gland when veratrine was injected. We have also been able to show that the extracts of veratrum viride are capable of increasing the release of epinephrine. The observation by Wood (16), Cramer (17), Pilcher and Sollmann (18), and Mac Nider (19) that an increase in blood pressure and an acceleration of heart rate occur in vagotomized animals after repeated doses of extracts of *veratrum viride* finds its explanation at least in part in this action.

The formation of an ester of cevine with a simple organic acid like veratric acid not only increases the effect of the alkaloid upon the mammalian and frog heart, but also imparts to it the ability to increase the depressor vagal influences upon the circulation, to act as a powerful respiratory depressant, and to cause, or at least enhance, the release of epinephrine.

SUMMARY

The qualitative and quantitative differences between cevine and its veratric acid ester, veratridine, on the isolated frog heart, on the blood pressure and respiration of the cat, and in unanesthetized mammals have been studied.

Cevine and veratridine cause reversible systolic standstill of the isolated frog heart and, in lower concentrations, improve the activity of the frog heart made hypodynamic by perfusion with calcium-poor salt solution. In confirmation of what has been observed in the isolated and denervated mammalian heart, there are only quantitative differences in the heart action of the two substances.

The primary effect of cevine in mammals is a convulsant one with no appreciable action on the circulation, while that of veratrine is a profound respiratory depression and a marked circulatory action caused chiefly by vagal stimulation.

Veratridine increases the liberation of epinephrine from the suprarenal glands. This constitutes at least one factor in the increase in blood pressure seen in the spinal animal after administration of cevine esters.

The qualitative effects of veratridine are indistinguishable from those of veratrine.

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CHRONIC SELENIUM POISONING IN DOGS AND ITS PREVENTION BY ARSENIC[1] [2]

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A number of papers have been published concerning the effect of chronic selenium poisoning on growth, food consumption, reproduction, and pathology of several species of laboratory animals (1, 2, 3) Little is known about the effects of chronic selenosis on the blood constituents (except hemoglobin), partially because of the limited quantity of blood which can be taken from small laboratory animals such as the rat, rabbit, and cat Therefore it seemed advisable to study the effects of chronic selenium poisoning on the blood picture of larger animals Dogs were selected for this purpose as convenient sources of blood samples sufficiently large to be analyzed for most of the commonly determined constituents Dogs are also large enough to furnish, at autopsy, samples of various organs and tissues large enough to be analyzed for selenium

The second part of this study was undertaken to determine the effect of arsenic when fed to dogs receiving a ration which contained selenium It had been found that arsenic would prevent or counteract the effects of selenium in rats (4, 5, 6) Later work has shown that arsenic has the same beneficial effects when fed to pigs receiving a seleniferous ration (7)

Recently a report on the effect of acute selenium poisoning in dogs was made from this laboratory (8), but there are no reports in the literature describing chronic selenosis of dogs

Experimental Thirty four dogs have been used in this work Fourteen dogs were used in the first part of the study between September 1935 and October, 1936 primarily to determine the effect of a seleniferous ration and twenty were used in the second phase of the investigation to determine the effect of arsenic in addition to a seleniferous ration This work was conducted between March 1938 and January, 1940 The division of the dogs into groups and the outline of the experiment are summarized in table 1

Feeding experiments The basic ration fed the dogs was the same for all groups (except group VII) and has the following composition

Corn, yellow	72 per cent
Casein, commercial	18
Lard	5
NaCl	1
$CaCO_3$	1
$Ca_3(PO_4)_2$	1
Yeast (Northwestern powdered)	1
Cod liver oil	1

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[1] Approved for publication by the Director of the South Dakota Agricultural Experiment Station as contribution No 168 of the Journal Series

[2] A preliminary report on a portion of this work was presented at the One Hundred and First meeting of the American Chemical Society St Louis Mo, April 10, 1941

TABLE 1

Outline of experiments

GROUP NUMBER	DOG NUMBER	SEX	STARTING AGE	DATE STARTED ON EXPERIMENT	DAYS ON EXPERIMENT	SELENIUM IN RATION		ARSENIC IN WATER, P P M	REMARKS
						Source	p p.m		
I	1	M	144	11/25/35	207	none	0	0	Littermates brought into laboratory 10 weeks old, kept on control ration for 74 days No 570 corn contained 29 p p m Se Blood samples taken at weekly intervals
I	2	M	144	11/25/35	207	corn	20	0	
I	3	M	144	11/25/35	41	Na_2SeO_3	20	0	
II	4	F	72	11/22/36	150	none	0	0	Littermates, born and raised in laboratory. Fed control ration after weaning until experiment started No 570 corn used in toxic ration.
II	5	M	72	11/22/36	150	none	0	0	
II	6	M	72	11/22/36	150	corn	20	0	
II	7	F	72	11/22/36	150	corn	20	0	
III	8	F	150	6/28/36	150	none	0	0	Littermate dogs, born and raised in the laboratory Maintained on control corn from weaning until start of experiment No blood samples were taken from dogs in this group
III	9	F	150	6/28/36	150	Na_2SeO_3	10	0	
III	10	F	150	6/28/36	150	Na_2SeO_3	10	0	
III	11	F	150	6/28/36	125	Na_2SeO_3	10	0	
III	12	F	150	6/28/36	125	Na_2SeO_3	10	0	
III	13	F	150	6/28/36	100	Na_2SeO_3	10	0	
III	14	F	150	6/28/36	100	Na_2SeO_3	10	0	
IV	15	M	mature	3/22/38	163	none	0	0	Mongrel dogs brought into the laboratory All had been maintained on the control corn ration for at least 60 days before the start of the experiment The rations were the same throughout the exp't No 16 was an old dog and probably died of causes not related to the ration
IV	16	M	mature	3/22/38	43	corn	7 2	0	
IV	17	M	150	3/22/38	163	corn	7 2	5	
IV	18	F	150	3/22/38	163	corn	7 2	5	
V	19	F	60	3/30/38	189	none	0	0	Littermate dogs born and raised in the laboratory They were fed control corn after weaning until the start of the experiment The rations were the same throughout the experiment.
V	20	M	60	3/30/38	189	corn	7 2	0	
V	21	M	60	3/30/38	189	corn	7.2	5	
VI	22	M	60	10/19/38	260	none	0	0	Littermate dogs raised in the laboratory and maintained on control corn before the start of the exp't (Dogs Nos 22 and 25 were kept in individual cages throughout the exp't Nos 23 and 24 were kept together in one cage and Nos 26 and 27 were kept together in another cage Arsenic added to water of No 26 after 148 days on exp't The Se content of the rations varied for this group due to difficulty in securing corn with an appropriate Se content) From 0-80 days the ration contained 3 p p m Se 81-237 days 11 p p m and 238-260 days 13 p p m selenium
VI	23	M	60	10/19/38	260	corn	3-11-13	5	
VI	24	F	60	10/19/38	665	corn	3-11-13	5	
VI	25	M	60	10/19/38	260	corn	3-11-13	0	
VI	26	F	60	10/19/38	260	corn	3-11-13	0-5	
VI	27	M	60	10/19/38	135	corn	3-11	0	
VII	28	M	60	9/20/39	122	none	0	0	Littermate dogs born and raised in the laboratory Maintained on control ration from weaning to start of exp't The ration fed these dogs contained corn as the source of Se until Oct 24 After that time, wheat was put in the ration to bring the Se content up to 13 p p m After Dec 1, the casein was replaced by tankage
VII	29	M	60	9/20/39	122	corn + wheat		0	
VII	30	F	60	9/20/39	122	corn + wheat		0	
VII	31	M	60	9/20/39	122	corn + wheat		5	
VII	32	F	60	9/20/39	122	corn + wheat		5	
VII	33	F	60	9/20/39	122		0	5	
VII	34	F	60	9/20/39	122		0	5	

The ration was ground and mixed complete except for the yeast and cod liver oil, then heated in an autoclave at 15 pounds pressure for 30 minutes After the heated ration cooled, the yeast and cod liver oil were added and the whole remixed When young puppies were reared on this ration, they were given a daily supplement of 100 grams of fresh liver, as an additional source of the vitamins of the B complex, until they were about 90 days old This ration is especially well adapted for the study of selenium poisoning as the large percentage of corn allows easy control of the selenium content of the ration, when seleniferous corn is used as the source of selenium This ration was modified by replacing the casein with tankage for the dogs in group VII Careful autopsies were made and samples of tissues were taken for selenium analyses Arsenic analyses were also made on some of the samples

Six female dogs (group III) were fed the control ration plus 10 parts per million selenium as sodium selenite for periods of time varying from 100–150 days Tissues from these dogs were analyzed to study the effect of length of feeding period on selenium deposition in the body

Analytical methods Blood analyses were made on samples from the dogs in all groups except III and VII The samples were collected from a femoral artery at weekly intervals Sodium citrate was used as an anti coagulant

Cell counts were made by the method described by Osgood and Haskins (9) Cell volume (hematocrit) was determined by centrifuging 15 cc of blood in a graduated tube until no further packing occurred For groups I through V, hemoglobin was determined by the improved Newcomer method (10), for group VI, hemoglobin was determined by the method of Evelyn (11)

Calcium was determined in all samples by precipitation as the oxalate, and titration of the washed precipitate dissolved in 1 N sulfuric acid with standard permanganate This is essentially the method described by Meyers (12) Inorganic phosphorus was determined by the method of Benedict and Theis as described by Osgood (10), on all the samples except those from group VI For group VI the method of Bodansky (14, 15) was used Phosphatase activity was also determined by Bodansky s method (14, 15)

Protein free filtrates were prepared by the method of Folin and Wu (16) and non protein nitrogen was determined by Nesslerization, after digestion of 2 cc of the filtrate with sulfuric acid and hydrogen peroxide essentially as described by Koch and McMeekin (17)

Chlorides were determined by the method described by Osgood and Haskins (9) Magnesium was determined by the methods of Hirschfelder and Serles (19) and of Briggs (18)

Blood sugar was determined by the method of Folin (21), and by a modification of the method of Folin and Malmros (20) adapted to the photoelectric colorimeter

Creatinine was determined by the modification of the method of Folin and Wu (16) adapted to the photoelectric colorimeter Bilirubin was determined by the method of Malloy and Evelyn (22) Selenium was determined by the digestion distillation method (2), and arsenic by the method described by Klein and Vorhes (23)

RESULTS *Growth and food consumption* The effect of selenium on the growth and food consumption of dogs is similar to the effect observed on rats (2) There is a marked restriction of food intake by dogs receiving selenium in the ration, the amount of restriction apparently depending on the selenium content of the ration and the degree of poisoning produced in each individual case This restriction of food intake was especially marked in the case of dog No 3 This dog so restricted his intake of a ration containing 20 p p m of inorganic selenium (sodium selenite) that he nearly starved On the fourteenth day, in an effort to persuade this dog to eat more, he was given control ration and an amount of selenium equal to that in the food consumed by dog No 2 on the previous day The selenium was given as a starch triturate in a capsule The dog continued to refuse to eat, apparently still associating the ration with the selenium. On the 35th day he was given an entirely new ration (commercial Dog Chow), and the selenium was administered in a capsule as before. For a few days he ate a moderately large amount of chow, then again refused to eat. This dog died after only 41 days on the seleniferous ration. Dogs receiving only 10.0 p.p.m. selenium as Na_2SeO_3 did not show such marked restriction of food intake. However, 10 p.p.m. of selenium was definitely toxic, as shown by the growth curves for group III (fig. 1).

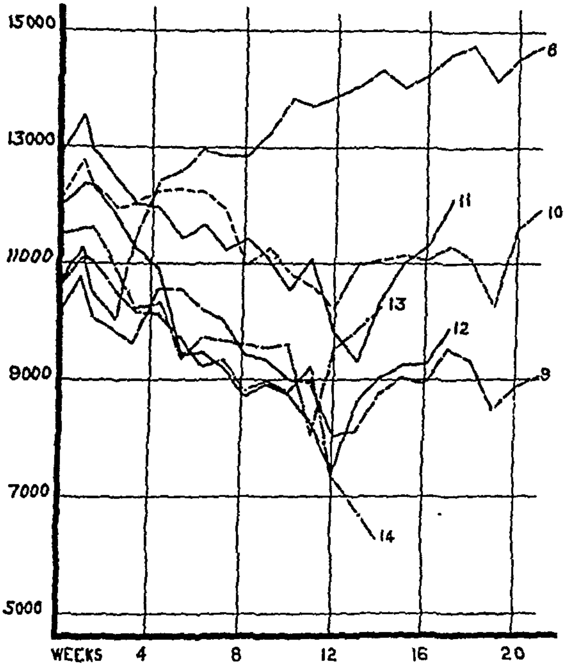

FIG. 1. GROWTH CURVES OF DOGS IN GROUP III (IN GRAMS)

Dog 8, control; dogs 9 to 14, 10.0 p.p.m. inorganic selenium for varying periods. See table 1.

When the dogs received the selenium as the naturally occurring form in grain, there was a restriction of food intake which became more severe as the degree of poisoning progressed. However, in such cases the dogs did not completely refuse to eat until the last day or two before death. When the dogs were given arsenic in the drinking water in addition to the seleniferous ration, there was no restriction of food intake. The food consumption records for group V (table 2) show the typical restriction of food intake by a dog receiving a ration with a low selenium content and the effect of arsenic in preventing this restriction.

The effect of selenium on the growth of dogs is well illustrated by the records for groups I and II which are typical. Table 3 shows the weight of the dogs in group I at the beginning and end of the experiment and at the time No. 3 died

(41st day of experiment) Dog No 2 reached a maximum weight of 14 45 kilograms on the 69th day of experiment and then gradually lost weight until the end of the experiment The growth curves for the dogs in group II are shown in figure 2

TABLE 2

Food consumed, grams per kilogram body weight per day[1]—Group V

DAYS ON EXPERIMENT	DOG NO 19 CONTROL RATION	DOG NO 20 SELENIFEROUS RATION	DOG NO 21 SELENIFEROUS RATION PLUS ARSENIC
10	71 4	35 1	55 4
20	58 4	42 6	45 7
30	64 5	25 3	56 4
45	43 5	18 8	46 8
50	39 8	26 0	36 8
60	37 4	18 9	38 1
70	34 9	18 0	34 7
90	30 3	24 1	32 6
100	29 8	17 9	26 4
110	28 2	10 3	24 2
130	24 3	14 3	24 8
140	24 7	18 6	25 4
155	23 5	16 7	23 9
160	15 5	19 3	18 2
170	21 3	13 1	20 2
180	18 2	14 3	15 0
189	19 8	9 5	29 6
Average	35 0	20 2	32 7

TABLE 3

Weight of dogs in Group I

DOG NUMBER	RATION	INITIAL	WEIGHT	
			41 days	Final (207 days)
			kgm	*kgm*
1	Control	15 5	18 0	20 9
2	Selenium (corn)	14 2	12 5	9 1
3	Selenium (Na_2SeO_3)	14 2	12 6	

Dogs which were given arsenic with either the seleniferous or the control ration grew normally, as shown by the growth curves for groups VI and VII (figs 3 and 4). In the former group, the control (No. 22) weighed 3.4 kilograms at the start of the experiment and 20.2 kilograms at the end. The corresponding weights for the dogs receiving arsenic plus selenium were: (No. 23) 2.7 kilograms and 25.1 kilograms, and (No. 24) 4.3 kilograms and 23.3 kilograms.

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[1] The average total food consumption per day for each period was calculated from the total amount eaten in the period and the number of days in the period The weights of the dogs at the beginning of the period and at the end of the period were averaged, and this average was used as the weight of the dog throughout the period It is recognized that this is only an approximation but when short periods (7 to 10 days) are used it is believed that a basis of comparison sufficiently accurate for the purpose is obtained

The dog which received the seleniferous ration alone (No. 25) weighed 4.5 kilograms at the beginning of the experiment and only 18.9 kilograms at the end of the experiment. This difference is especially noteworthy as dog No. 25 had a much larger skeleton than any of the other dogs. On the 187th day ascites was obvious, and on the 200th day 1.34 kilograms of ascitic fluid were drained from the abdominal cavity; on the 226th day an additional 4.39 kilograms of fluid were removed (see fig. 3).

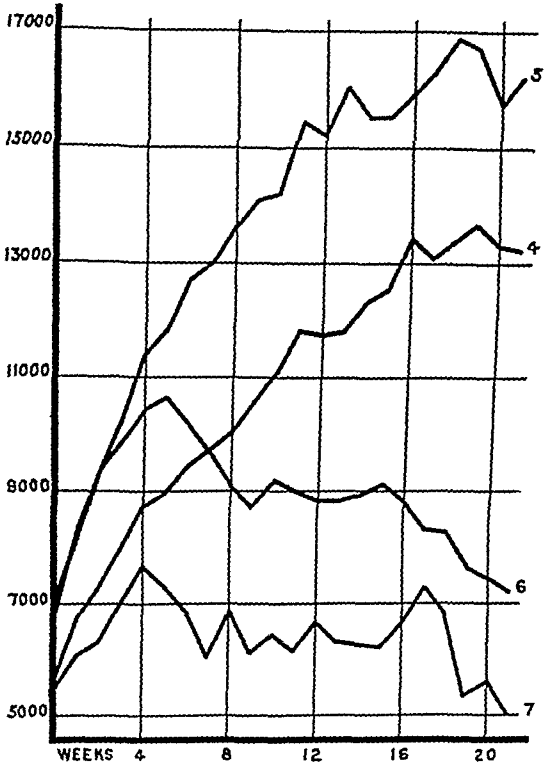

FIG. 2. GROWTH CURVES OF DOGS IN GROUP II (IN GRAMS)
Dogs 4 and 5, control; dogs 6 and 7, 20 p.p.m. selenium (seleniferous corn). See table 1

Dog No. 27 weighed 3.9 kilograms at the beginning of the experiment and only 11.5 kilograms when she died on the 135th day, as compared with 21.2 kilograms for the control dog (No. 22) on the same day. Dog No. 27 did, however, weigh 15.4 kilograms at 120 days. Ascites was noted in this dog at the

90th day and on the 125th day 4.17 kilograms of ascitic fluid were drained from the abdominal cavity (see fig. 3)

The case of dog No. 26 is especially interesting. This dog weighed 4.8 kilograms at the beginning of the experiment and only 13.5 kilograms at the end of 135 days on the seleniferous ration, as compared with the control dog (No. 22) which weighed 21.2 kilograms at that time. On the 148th day 5 p.p.m. of arsenic (sodium arsenite) were added to the drinking water for dog No. 26. At the end of the experiment (260 days), this dog weighed 2.4 kilograms more than the control dog. There was no evidence of ascites, and in this case the arsenic apparently effected a satisfactory recovery from a rather severe case of chronic selenium poisoning (dog No. 26, fig. 3). In experiments on selenium-arsenic antagonism in rats (6), the arsenic was of little value in treating rats which had been fed selenium for more than 30 days at the time the arsenic treatment started, but was effective for rats which had been fed selenium for not more than 20 days at the beginning of the arsenic treatment.

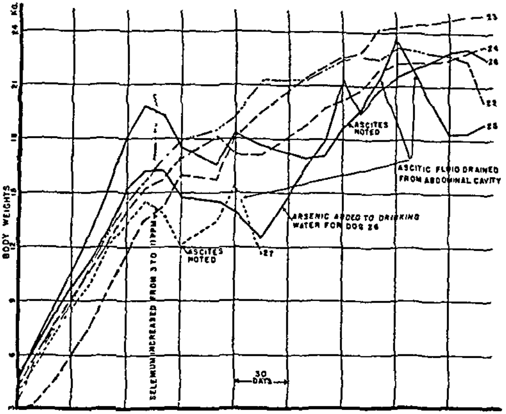

FIG. 3. GROWTH CURVES OF DOGS IN GROUP VI

Note effect of selenium on growth of dogs 25 and 27, and the effect of selenium + arsenic on dogs 23, 24 and 26 See table 1.

*Since arsenic is a poisonous element, it seemed desirable to feed it with a sele*nium-free ration at a level of 5 p p.m. in the drinking water to observe its effects in the absence of selenium. Two dogs (No. 33 and No. 34) in group VII were fed arsenic at this level and no adverse effect on growth (fig. 4) or appearance of these dogs was noted. This is in agreement with the observation of Calvery, Laug, and Morris (24) that dogs fed a ration containing relatively high levels of arsenic as the trioxide were comparable to the controls.

The dogs in group VII which received the seleniferous ration plus arsenic grew satisfactorily (No. 31 and 32), while those fed the seleniferous ration without arsenic (No. 29 and No. 30) grew poorly. Ascites was very severe in both dogs which were fed the seleniferous ration without arsenic. The amounts of ascitic fluid removed at autopsy are shown in figure 4.

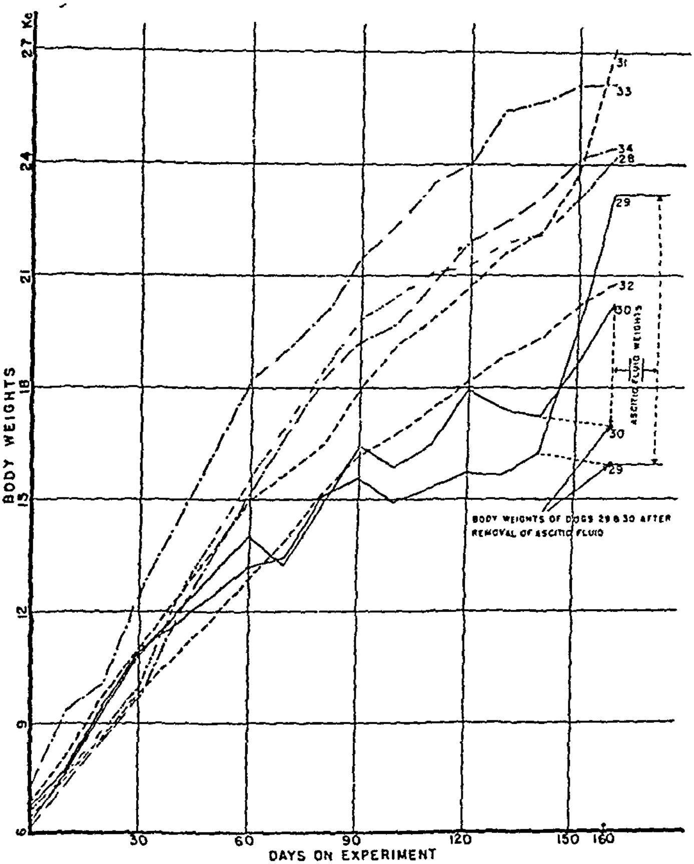

Fig. 4. Growth Curves of Dogs in Group VII

Note effect of selenium on dogs 29 and 30, of selenium + arsenic on dogs 31 and 32, and of arsenic on 33 and 34. Dog 28 was the control. See table 1.

One dog (group VI, No. 24) was fed selenium plus arsenic for 22 months. This dog remained in excellent health and at autopsy, after being sacrificed, showed no harmful effects. In contrast, one of her litter-mates (No. 27) fed selenium without arsenic died on the 135th day of the experiment (fig. 3).

Much individual variation in susceptibility to poisoning by selenium was noted Young dogs were much more susceptible than older dogs These observations have been noted with other species (25)

CONDITION OF THE DOGS AND LESIONS NOTED AT AUTOPSY In all the groups studied the control dogs showed no abnormalities at autopsy Several typical effects of selenium intoxication were apparent in the dogs which were fed only the seleniferous rations The dogs fed the seleniferous rations plus arsenic resembled the controls From the records of all the dogs the typical external appearance and autopsy findings may be summarized as follows

Chronic selenium poisoning Due to reduced food intake the animals became markedly emaciated, their hair coarse and loose Frequently the abdomen filled with ascitic fluid with or without an accompanying edema of the feet Walking was often difficult or painful When the nervous system was severely affected the dog's response to commands or noises was very slow, the eyes became dull and lifeless in appearance Some of the typical symptoms of "blind staggers" (26) were exhibited In such instances the dogs walked blindly and often stumbled over objects in their path

There was very little abdominal or peritoneal fat (The viscera were very pale in color due to the severe anemia) The kidneys were usually not visibly affected, but the adrenals were atrophied The spleen was enlarged and had localized hemorrhagic areas The ovaries or testes were very poorly developed

In nearly all cases of selenium poisoning the liver was atrophied (especially the central lobe), necrotic, cirrhotic (nodular), and highly pigmented in localized areas This condition is shown by the livers of Nos 6 and 7 (fig 5) and also the portion of liver shown in figure 6, dog No 29

Histological examination[4] of some of the typical livers revealed moderate fatty degeneration, and nodular cirrhosis with an excess of interstitial connective tissue and periportal hemorrhages Obstruction of the bile duct was evident in severe cases and the bile varied from normal color and viscosity to deeply yellow green and very viscous

Small, localized hemorrhages were present in the intestines of some of these dogs In the others the stomach and intestines appeared to be normal The appearance of the heart varied from normal to soft and flabby ("dish rag") (26)

Ascites developed in most of the dogs receiving selenium This condition was always accompanied by a dilatation of blood vessels in the kidney region as shown in figures 6 and 7 The specific cause of this dilatation is not now known The ascites appear to be associated with some decrease in plasma protein

Seleniferous ration plus arsenic The external appearance of all of the dogs fed arsenic in addition to the seleniferous ration was normal in all respects and they were more alert and active than the control dogs The hair was soft and glossy Upon autopsy the abdomen was covered with a thick layer of subcutaneous fat and there was an abundance of peritoneal fat

There were no gross lesions The liver was normal in size, smooth, and clear

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[4] All histological examinations were made by Dr J B Taylor Experiment Station Veterinary Pathologist

Histological examination revealed slight fatty degeneration, but no excess of interstitial connective tissue or other evidence of cirrhosis. There was, however, an abundance of dark colored intracellular deposits in the livers. The gall bladder was normal. The ovaries or testes were well developed.

Selenium-free ration plus arsenic. These dogs were normal in all respects and showed the same distribution of fat as the dogs fed arsenic in addition to the seleniferous ration.

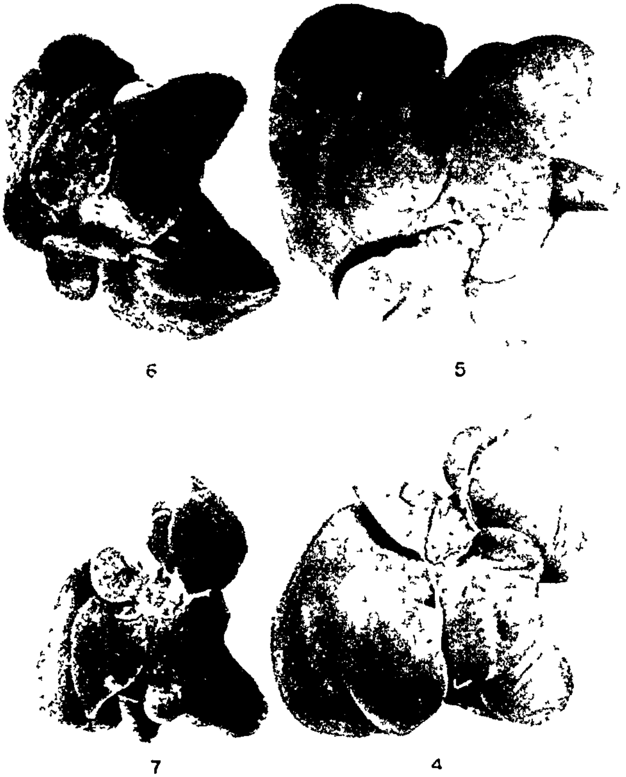

Fig 5 Livers from Dogs in Group II

(Nos correspond to dog Nos) Note cirrhosis, and pigmentation of livers from selenized dogs (6 and 7) See table 1.

EFFECT OF SELENIUM ON THE BLOOD PICTURE The large amount of data collected from the analyses of blood samples from the dogs show that chronic selenium poisoning has little or no effect on the commonly determined constituents, except hemoglobin and possibly *non-protein nitrogen and phosphatase activity*

The depression of the hemoglobin level in rats fed selenium over a period of time has been reported (27) That the same effect occurs in dogs is shown by the data from group VI, dogs Nos 25 and 27 (fig 8) In the same group the data from dogs Nos 23 and 24 show the effect of arsenic administration in maintaining hemoglobin near the normal level The data from dog No 26 (fig 8) indicate that arsenic can bring about partial recovery from the anemia caused by chronic selenium poisoning

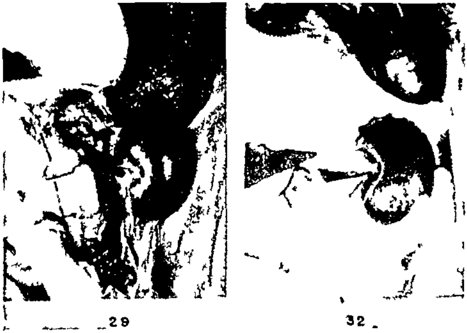

FIG 6 SHOWING KIDNEY REGIONS OF DOGS FED SELENIUM (29) AND SELENIUM + ARSENIC (32)

Note dilatation of blood vessels in kidney region and cirrhotic condition of liver in dog 29

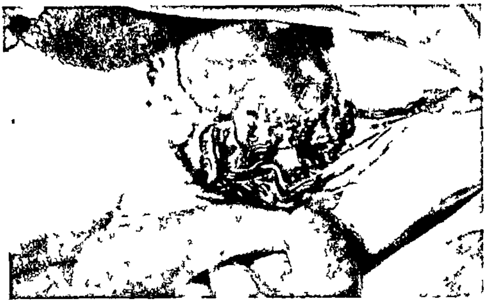

FIG 7 DILATATION OF BLOOD VESSELS IN KIDNEY REGION OF DOG 30

Additional data from groups I and II show that as the hemoglobin level decreases the red cell count decreases proportionally This would suggest that the anemia is due to sub normal formation of red cells (oligocythemia) rather than to decreased hemoglobin content of the cells which are formed. A tendency for lower non-protein nitrogen values in the dogs fed seleniferous rations was noted in all groups Although the differences are not great, they are consistent and are probably associated with the voluntary restriction of food consumption.

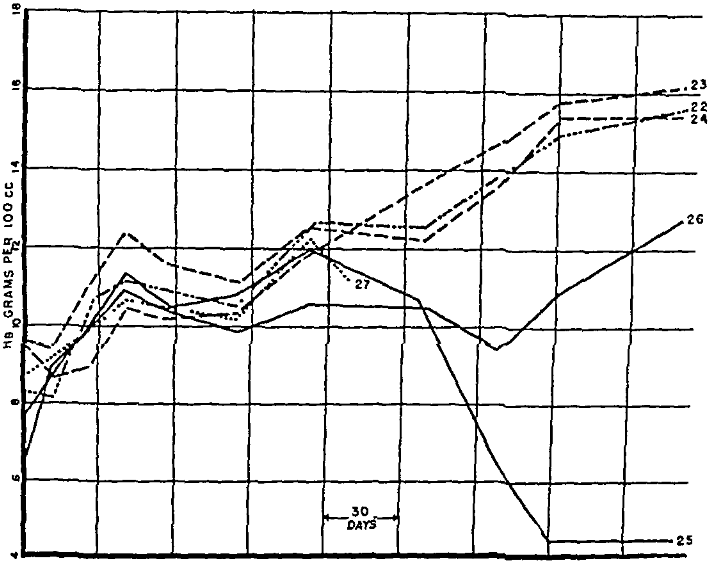

FIG 8 HEMOGLOBIN LEVELS OF DOGS IN GROUP VI

Note effect of arsenic in maintaining hemoglobin level of dogs 23 and 24, and recovery of dog 26 after administration of arsenic from the 148th day

TABLE 4

Phosphatase activity of plasma—Group VI

DAYS ON EXPERIMENT	DOG NUMBER AND TREATMENT				
	22 Control	23 Se + As	24 Se + As	25 Se	26 Se Se + As
169	11 35	7 11	10 39	28 84	22 18
183	6 32		8 96	34 47	14 38
193	7 24	8 42	7 86	10 74	12 34
208	8 54	6 50	6 16	7 63	7 92
243	2 35	3 90	2 70	12 58	5 08
260	2 74	2 56	2 78	17 58	5 78

In general, the values for creatinine paralleled the values for total non-protein nitrogen in the blood.

The data obtained on blood phosphatase activity are limited (table 4) However, the very marked difference between the control (No 22) and the selenium-arsenic dogs (Nos 23, 24, 26) and the dog receiving selenium alone (No. 25) is striking. It has been reported that phosphatase activity is high in some cases of hepatic cirrhosis (28). The action of arsenic in preventing (in dog No 26) the rise in phosphatase exhibited by dog No. 25, when the selenium content of the ration was increased on the 237th day of the experiment, is noteworthy.

The data indicate that the rest of the blood picture is not markedly affected by seleniferous rations No effort was made to control the eating habits of the dogs, and week to week variations in the blood sugar, calcium, phosphorus, and magnesium would make it impossible to observe any slight changes in levels of these constituents due to the ration.

Generally a decrease in calcium, phosphorus, and magnesium of the blood was observed to occur as the dogs reached maturity.

In acute selenium poisoning (8) a marked rise in hemoglobin was noted. This is in contrast to our findings in chronic selenosis. Other effects on the blood picture were likewise more marked in acute poisoning.

DISTRIBUTION OF SELENIUM AND ARSENIC IN THE TISSUES. The analyses of various body tissues for selenium (table 5) show that while the liver and kidney usually contain the greatest amounts of selenium, all the organs of the body retain some In general, the liver and kidneys from the dogs of group 2, which were given 10 p.p m. selenium as Na_2SeO_3, contained more selenium than the same organs from dogs fed an equal amount of selenium as seleniferous grain.

It is interesting to note that the brains from dogs Nos. 6 and 26 contained 10 p p.m. of selenium, much more than the brains from any of the others. These two dogs showed the greatest effect of poisoning on the nervous system.

There is no apparent relationship between the selenium content of the tissues and the length of time the dogs were fed the seleniferous rations. Neither is there any consistent reduction in selenium content of the tissues of the dogs fed arsenic along with selenium It is interesting to note that Fairhall and Miller (29) observed a marked reduction in the deposition of lead in body tissues in the presence of arsenic as compared with the amount of lead deposited in the absence of arsenic.

The arsenic content of the tissues of the dogs of group VII is shown in table 6. Arsenic, like selenium, finds its way into all tissues, and there are no consistent differences in concentration in the various organs This is in accord with the findings of Harkins and Swain (30) in their investigation of chronic arsenical poisoning of livestock. Much individual variation in deposition of both selenium and arsenic was noted

In our investigations, dogs have exhibited many of the symptoms of selenium poisoning previously described for farm animals more markedly than other animals that have been used in laboratory experiments. Severe nervous effects, with symptoms resembling "blind staggers" were observed in two of the dogs. The appearance of the more severely affected dogs strongly resembled that of cattle, horses, and pigs that are lame, thin, and have lost hair due to chronic selenosis. The effects of arsenic in preventing and counteracting selenium poisoning in the

TABLE 5

Selenium in body tissues (mgm. per kgm. dry wt.)

DOG NUMBER	SEX	FORM OF SELENIUM IN RATION	DAYS ON EXPERIMENT	TOTAL MGM. SELENIUM CONSUMED	LIVER	KIDNEYS	HEART	SPLEEN	LUNGS	INTESTINES	BRAIN	PANCREAS	BLOOD CELLS	MUSCLE	BONES
2	M	20 p.p.m. corn 570	207	634.7	36.6	26.6	10.0		7.0			24	0.15		
3	M	20 p.p.m. Na_2SeO_3	46	23.95	12.0	32.0	2.66	5.0	3.3	1.7	*	9.0	0.40	*	0.3
6	M	20 p.p.m. corn 570	151	488.6	23.3	40.0	16.6			10.0	10	15.4		10.0	ribs 22.6 long bones 2.45
7	F	20 p.p.m. corn 570	151	430.6	43.3	66.6	13.3	23.3		8.33		25.0		6.7	*
9	F	10 p.p.m. Na_2SeO_3	150	203.89	30.0	20.0			*	trace	trace	0.6	0.40	*	
10	F	10 p.p.m. Na_2SeO_3	150	354.41	33.0	27.0	0.6	trace	5.0	0.6		trace	0.16	*	
11	F	10 p p.m. Na_2SeO_3	129	271.73	20.0	6.0	0	3.0	6.5			*	*	*	
12	F	10 p.p.m. Na_2SeO_3	129	237.08	67.0	26.0	2.0	*	3.0	trace		trace	*	2.0	
13	F	10 p.p.m. Na_2SeO_3	100	110.41	26	20.0	trace	3.3	*	*	trace	*			
14	F	10 p.p.m. Na_2SeO_3	100	117.84	20.0	20.0	trace	*	3.3	2.0	*	*		1.7	
16	M	corn	163	41	7.0	10.3	2.9	14.3							
17	M	corn	163	253	36.0	8.3	11.8	7.9	6.4		*		7.1		
18	F	corn	163	275	12.6	8.1	6.2	9.8	11.4	+ stom. 15	3.8				*
20	M	corn	189	219	12.5	72.0	4.0		4.0	+ stom. 8.31	4.5	16.0	10.0		1.3
23	M	corn	260	1,102	35.0	6.0	12.0	2.5	*	stom. 10, 3.5	4.4	*	1.5	14.0	*
24	F	corn	665		12.0	4.0	0.5	3.1	*	*	*	*	0.3	2.1	2.0
25	M	corn	260	734	15.0	15.9	12.0	5.8	12.8	stom. 5.9, 4.8	6.2	4.9	0.8	8.5	0.9
26	M	corn	260	1,101	37.5	32.0	8.0	10.0	7.5	stom. 2.5, 4.0	10.0	4.0	*	*	*
27	F	corn	155	196	16.0	20.0	11.0	15.3	9.0	stom. 7.0	3.0	3.2		3.0	3.0
29	F	corn, wheat	122		16.5	11.9	1.2	2.8	0.90	0.8	1.2	0.7	0.2	1.6	2.7
30	M	corn, wheat	122		4.9	21.6	2.3	1.6	1.7	1.1	1.2	1.3	0.3	2.3	2.4
31	F	corn, wheat	122		2.6	6.1	4.3	1.7	1.2	0.6	1.0	1.3	*	2.3	8.7
32	M	corn, wheat	122		10.4	3.4	5.6	5.5	0.5	1.9	0.7			7.1	4.5

* Less than 0.005 mgm. selenium in sample. Sample weights varied from 5 0 to 10.0 grams.

TABLE 6

Arsenic content of tissues from dogs in Group VII

DOG NUMBER	SEX	DAYS ON EXPERIMENT	LIVER	KIDNEY	HEART	SPLEEN	LUNGS	INTESTINES	BRAIN	PANCREAS	BLOOD	MEAT	BONES
31	Male	122	1.4	0.6	0.1	0.5	0.1			0.8		1.3	7.6
32	Female	122	3.4	0.6	0.8	1.5	1.3	1.6	1.4	1.1		1.2	70.3
33	Female	122		3.6	1.5	2.4	2.3	2.5	1.3	9.1		2.6	
34	Female	122	1.6	1.4	0.7	2.1	2.2	3.8	1.3	3.0	0.5	1.5	33.0

dog appear to be the same as those observed for other animals (6, 7). It would seem from these observations that dogs are especially well suited for use in further detailed studies of selenium poisoning.

SUMMARY

A study of chronic selenium poisoning in dogs has been made using a basic ration high in grain. As little as 7.2 parts per million of selenium in the ration in the natural form and 10 parts per million as added sodium selenite were toxic as shown by sub-normal growth and restricted food intake.

Twenty parts per million of selenium as added sodium selenite induced refusal of food and death in a very short time; and in the natural form this amount of selenium produced severe nervous disorders.

The pathological symptoms of chronic selenium poisoning are described. The liver and spleen were the organs most severely affected. Severe ascites occurred in many cases. This was accompanied by extreme dilatation of blood vessels in the abdominal cavity.

Arsenic in the form of sodium arsenite added to the drinking water at the rate of 5 parts per million of the water was effective in counteracting or preventing the symptoms of chronic selenium poisoning, when the ration contained as much as 13 parts per million of selenium in the natural form. This amount of arsenic, when fed in addition to the control ration, produced no symptoms of poisoning, but was conducive to the deposition of subcutaneous and abdominal fat.

Both selenium and arsenic were found in all tissues of the body examined. The liver, kidney, and spleen contained the most selenium. There was no relationship between the length of time selenium was fed and the amount found in the various tissues. Individual variation was marked.

Chronic selenium poisoning has little effect on the commonly determined blood constituents except hemoglobin, and possibly phosphatase activity. The hemoglobin is markedly reduced depending on the severity of the poisoning in each individual case. Arsenic prevented the anemia caused by selenium poisoning. Phosphatase activity is increased in selenium poisoning. Non-protein nitrogen, in general, is lower in chronic selenium poisoning than in the controls, but not markedly so.

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DIHYDROXYPROPYL BISMUTHATE: EXPERIMENTAL STUDIES OF A NEW BISMUTH COMPOUND[1]

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The value of bismuth compounds as therapeutic agents has been established. Originally, their chief medicinal use was confined to their local actions. With the recognition of the value of bismuth in the treatment of syphilis, a large number of compounds, both soluble and insoluble, have been introduced for systemic medication. This trend has brought about the development of organic preparations which owe their efficacy to their bismuth content. Since most of these compounds are insoluble, or readily become insoluble when administered orally, they are administered by intramuscular injection. The purpose of this report is to describe a soluble organic compound of bismuth which is suitable for oral administration by virtue of appreciable systemic absorption. A review of the literature reveals that workers in this field have suggested several characteristics desirable in a bismuth compound to be used in oral medication, namely, that it be a pentavalent, electronegative bismuth combination, it should form aqueous solutions that are neutral or capable of being buffered, and be reasonably stable in the fluids of the body (1, 2, 3, 4, 5, 6). A compound fulfilling these essential characteristics, dihydroxypropyl bismuthate,[2] has been prepared by the carefully controlled esterification of glycerin and sodium bismuthate. The reaction takes place as follows:

$$C_3H_5(OH)_3 + NaBiO_3 \rightarrow C_3H_5(OH)_2BiO_3 + NaOH$$

The reaction mixture is neutralized with citric acid and the dihydroxypropyl bismuthate is isolated and purified by reprecipitation in ethyl alcohol. This preparation is very soluble in water, stable in mild acidity and alkalinity, and compatible with body fluids It contains 62 80% of metallic bismuth

PHARMACOLOGICAL STUDIES *Stability of Dihydroxypropyl Bismuthate in Gastric Juice.* While dihydroxypropyl bismuthate is stable in mild acidity it is unstable in the presence of approximately 2% of a mineral acid It was deemed advisable to determine *in vitro* the behavior of dihydroxypropyl bismuthate in gastric juice The juice used was obtained from dogs with Pavlov pouches and collected after stimulation of the gastric secretion by intramuscular injection of histamine. The juice was almost water clear, having had all particles removed by filtration When drawn, the pH of the juice was 1 1, but after being mixed with the bismuth solution, the pH rose to 2.8, as determined by the glass electrode potentiometer.

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[1] Presented in partial fulfillment of the requirements of the Graduate College of the State University of Iowa for the Ph D Degree, April, 1943

[2] The preparation and chemical properties are described in a Master's thesis submitted to the Graduate College, State University of Iowa, June, 1941

In order to determine the amount of precipitation of bismuth over increasing lengths of time, six tared centrifuge tubes were arranged so that each contained 5 cc. of gastric juice and 150 mgm. of dihydroxypropyl bismuthate. After periods of time varying from 30 minutes to 24 hours, the tubes were centrifuged to settle any bismuth rendered insoluble, decanted and the precipitate washed once with water. After drying at 100° C., the tubes were reweighed and the increase considered to be bismuth oxychloride.

As can be seen in table 1, dihydroxypropyl bismuthate is almost completely stable in gastric juice at a pH of 2.8. The period of experimentation indicates stability in the gastric juice much longer than the normal sojourn in the stomach. Hence the molecule should remain intact and be water soluble as it reaches the intestine. The duration of solubility in the intestines will probably vary with the rate of sulfide formation.

Toxicity Determinations. Intramuscular: The rate of absorption from an intramuscular injection is largely determined by the physical and chemical properties of any bismuth preparation (4). Hence a stable, water soluble bismuth compound would show both the highest rate of absorption and toxicity. Since dihydroxypropyl bismuthate is soluble both in water and in glycols miscible with water, these vehicles present desirable solvents for intramuscular administration.

TABLE 1

Stability of dihydroxypropyl bismuthate in gastric juice

TIME	WT. OF PRECIPITATE	% TOTAL BISMUTH PRECIPITATED
hours	*mgm.*	
½	3.7	1.97
1	4.0	2.10
4	5.5	2.90
8	6.3	3.36
12	7.1	3.79
24	7.5	4.00

In an effort to determine the maximum tolerated dose by intramuscular injection, 73 rats were given series of doses intended to exceed the average minimum lethal dose. Two parallel series were arranged to compare the minimum lethal intramuscular dose using an aqueous solution and a solvent prepared from equal parts of water and propylene glycol. Fifty-seven of the above rats weighing from 150 to 250 grams received intramuscular injections of an aqueous solution of dihydroxypropyl bismuthate. This experimental solution contained 3% trisodium citrate which raised the pH from 6.9 to 7.2. Doses ranged from 12 to 50 mgm. of bismuth per kgm. Twenty-nine rats receiving 30 mgm. or less per kgm. all survived; of 4 rats receiving 32 mgm. per kgm., 1 died; and all rats receiving 34 mgm. or over died. Deaths occurred in 2 to 4 days after injection. With this limited series of animals, the minimum lethal dose was established at about 32 mgm. bismuth per kgm.

A second series of 16 rats of about the same size were injected intramuscularly with increasing doses of dihydroxypropyl bismuthate in equal parts of water and propylene glycol. This solution also contained 3% trisodium citrate. Twelve rats receiving 10 to 35 mgm. per kgm., all survived; of 2 rats receiving 40 mgm., one died; and both rats receiving 45 mgm. died. Propylene glycol definitely slows absorption, as evidenced by increase in the minimum lethal dose as compared to the aqueous solution. The lethal effects in both series appeared approximately 72 hours following the injection.

Oral: In an attempt to establish a toxic oral dose of dihydroxypropyl bismuthate, 35 rats and 11 rabbits were used. Systematically arranged doses were administered by stomach tube. The aqueous solution contained 3% trisodium citrate. The rats were given doses ranging from 300 to 1,250 mgm. of bismuth per kgm. The increment in dosage was 25 mgm. per kgm.; dosages above 1,250 mgm. per kgm. were not employed. All rats survived without showing any symptoms of toxicity.

Using a solution of the same composition as employed for rats, 9 white rabbits were administered oral doses ranging at 100 mgm. increments from 300 to 1,400 mgm. of bismuth per kgm. All animals in the series receiving dosages of 1,000 mgm. bismuth per kgm. or more, died, and the survival time varied inversely with the dosage.

Intravenous. Twenty-two rats were given dosages ranging from 5 to 15 mgm. of bismuth per kgm. Injections were made into the saphenous vein without undue difficulty. All the rats receiving 7 mgm. or less per kgm. survived, of two rats receiving 8 mgm , one died. All dosages above this amount were fatal. The deaths occurred from 1 to 7 days after the injection, with the survival time varying inversely with the dosage. The L D.^{50} for intravenous injections in rats was established at 8 mgm

Of 5 white rabbits, two received the compound in dosage of 5, two 10, and one 20 mgm. bismuth per kgm injected into the marginal ear vein. Since all died except one receiving 5 mgm., rabbits appear to be less resistant than rats to the intravenous administration of dihydroxypropyl bismuthate Deaths occurred from 2 to 7 days after administration, the time of survival varying inversely in proportion to dosage

Twenty-four hours after the injection of the rabbit receiving 20 mgm. bismuth per kgm., a blood sample was drawn and analyzed The blood contained 1.0 mgm per cent bismuth which is a remarkably high concentration. The time elapsing between injection and death is very suggestive of kidney damage and inability to eliminate bismuth may account for the high blood level in the analysis cited.

METHOD OF DETERMINING BISMUTH IN BIOLOGICAL MATERIAL. In general, the method described by Leonard (7) and modified by Weigand (8) was adapted to both milligram and microgram sensitivities The color was determined by using a Fisher electro-photometer, by means of which differences of less than one microgram can be detected Standard calibration curves in terms of percentage absorption were prepared using electrolytic bismuth metal dissolved in nitric acid.

Urine A suitable, acidified aliquot was concentrated by boiling and oxidized with potassium permanganate. Oxalic acid and filtration were employed to clarify the sample before developing the colored bismuth complex.

Feces, Blood and Organs: These fractions were dried and ignited below 500°C. in a muffle furnace. The inorganic residue was then dissolved in sulfuric acid and the bismuth color developed.

Carcass: Ignited as the feces, the carcass residue must be first taken into complete solution in hydrochloric acid from which the bismuth is precipitated as the sulfide. This precipitate is then dissolved in sulfuric acid and the color developed.

Brain and Spinal Fluid: These substances are wet ashed by the Newmann method and the potassium iodobismuthite measured colorimetrically.

Using the described oxidation methods on the various tissues and fluids with known amounts of bismuth added, the following recoveries were obtained (table 2).

TABLE 2

Recovery of known amounts of bismuth from various biological materials

SUBSTANCE	BLANK DET'N	BI. ADDED	BI. RECOVERED	AV. ERROR
	mgm.	*mgm.*	*mgm.*	%
Urine	0.02	1.0 5.0 10.0	1.01 4.93 9.96	1.5
Feces	0.03	1.0 5.0 10.0	0.92 4.90 9.88	3.0
Carcass	0.12	2.0 10.0 25.0	1.91 9.80 24.20	3.2
Blood	0.0004	0.0025 0.0100 0.0500	0.0027 0.0102 0.0492	1.6
Brain and spinal fluid	0.0004	0.0025 0.0100 0.0500	0.0026 0 0100 0.0496	2.2

URINARY AND FECAL ELIMINATION OF BISMUTH FOLLOWING ADMINISTRATION OF DIHYDROXYPROPYL BISMUTHATE. For excretion studies, albino rats were placed in metabolism cages over funnels arranged to collect urine and feces separately. The feces were retained more or less uniformly dispersed upon a lacquered copper platform resting near the top of the funnel. This platform was perforated and paraffined to allow the urine to pass freely into the receiving graduate. Keeping the feces dispersed prevented contamination of the urine. A second fine screen was placed in the apex of the funnel to prevent contamination of the urine by fine fragments passing through the upper copper plate. Contamination from food was minimized by mixing ground "Friskies" with lard to form a coherent paste.

Oral Administration: Four rats each were used to follow bismuth excretion from a single oral and from chronic oral administration. Single doses were intended to give an indication of the relative duration of the urinary and fecal elimination of bismuth The chronic dosage was used to determine what level of excretion of bismuth occurs in the urine from continued administration Four rats were used to demonstrate that bismuth was present in the urine several days after a single oral dose of 50 mgm of bismuth in the form of dihydroxypropyl bismuthate A large percentage of the bismuth was found in the feces at the end of the first 24 hours, but thereafter the fecal elimination was very small It is demonstrated that the bulk of the administered bismuth, some of which was yet soluble, left the body unabsorbed Urinary excretion of bismuth was determined at 48 hour intervals during the daily oral administration of 50 mgm of dihydroxypropyl bismuthate per rat

TABLE 3

Excretion of bismuth from a single oral dose

DAYS[1]	RAT NO 1		RAT NO 2		RAT NO 3		RAT NO 4	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
	mgm	*mgm*	*mgm*	*mgm*	*mgm*	*mgm*	*mgm*	*mgm*
1	0 4	40 8	0 6	41 3	0 5	40 5	0 6	42 6
2	0 7	5 3	0 8	3 8	0 5	4 2	0 5	3 8
3	0 75	0 6	0 9	0 4	0 4	0 8	0 4	1 1
4	0 6	0 1	0 8	Trace	0 6	0 2	0 3	0 8
5	0 3	None	0 3	None	0 2	Trace	0 1	0 3

TABLE 4

Urinary bismuth excretion following continuous oral administration
Mgm bismuth excreted daily

DAYS	RAT NO 1	RAT NO 2	RAT NO 3	RAT NO 4
1	1 0	1 2	0 5	0 5
3	1 3	1 2	0 8	0 6
5	0 6	0 8	0 9	0 9
7	1 1	0 9	1 3	1 2
9	0 8	0 7	1 2	1 3
11	0 9	1 1	1 4	1 5
13	1 3	1 5	1 2	1 25
15	1 0	1 2	1 3	1 35
17	0 8	1 1	1 35	1 25
19	1 1	1 3	1 1	1 5

As would be expected, the continued oral administration resulted in the continued urinary elimination of bismuth The range of absorption, based upon urinary excretion was approximately 2% as demonstrated when four rats were employed As indicated by table 4 the bulk of the bismuth was eliminated in the feces which were black as a result of the formation of bismuth sulfide

Intramuscular Administration Four rats were used to determine the bismuth excretion following continued intramuscular administration of dihydroxypropyl bismuthate. At 3 day intervals each of four rats received 15 mgm. of bismuth per kgm. by intramuscular injection. All rats appeared to remain normal in every respect. No irritation nor discomfort was observed following the injections. The injections were continued at 3 day intervals for 24 days. The urine was analyzed in 72 hour specimens but the feces were omitted. As can be seen from table 5, dihydroxypropyl bismuthate is readily mobilized from the site of injection. Nearly 20% is execreted within 3 days following the injection compared to the 12 days necessary to mobilize approximately the same percentage of other compounds (9).

TABLE 5

Urinary excretion bismuth following chronic intramuscular injections
Mgm. bismuth excreted daily

DAYS	RAT NO. 1	RAT NO. 2	RAT NO. 3	RAT NO. 4
3	1.1	1.25	0.5	0.65
6	1.35	1.32	0.65	0.7
9	1.4	1.35	0.78	0.65
12	1.4	1.36	0.82	0.78
15	1.5	1.5	0.86	0.81
18	1.8	1.67	0.92	0.86
21	2.0	1.82	0.88	0.78
24	1.9	1.85	1.10	0.82

TABLE 6

Total recovery from a single oral administration of bismuth

RAT NO.	HOURS BEFORE SACRIFICED	DOSE	MGM. BISMUTH RECOVERED				
			Urine	Feces	G.I. Tract	Carcass	Total
		mgm. Bi.					
1	24	55	1.8	48.4	4.0	0.28	54.48
2	48	48	2.4	39.3	3.65	0.7	46.05
3	72	48	2.5	43.6	0.3	0.7	47.20

Fate of a Single Oral Administration of Dihydroxypropyl Bismuthate. Three rats were sacrificed for this purpose. They were killed after 24, 48, and 72 hours respectively, following the bismuth administration. All urine and feces were collected separately. The rats were eviscerated and the urine, feces, gastrointestinal tract with contents, and carcass were analyzed separately. By analyzing such fractions both the fate of a single oral dose and the relative rate and amount of gastric absorption can be determined, as all the bismuth administered should be recovered. That practically complete recovery is possible is demonstrated within narrow limits of experimental error as seen in table 6.

From these results it becomes evident that accumulation of toxic amounts of bismuth in vital organs is unlikely, and that damage should not exist even though the subject is absorbing and excreting significant amounts of bismuth daily.

This advantage can clearly be established over the accumulation of bismuth from intramuscular injection, both at the depot of injection and throughout the body (9, 10).

Bismuth in Brain Tissue of Rats Following Oral Administration of Dihydroxypropyl Bismuthate. Seventeen adult, male rats were given by stomach tube a solution of dihydroxypropyl bismuthate containing 3% trisodium citrate. Two daily doses of 50 mgm. bismuth each were given for 6 days. On the seventh day the rats were beheaded, the brains removed, washed and rapidly weighed. Subsequent analysis revealed unusually high concentrations of bismuth as seen in table 7.

TABLE 7

Bismuth content of brain tissue following oral administration

RAT NO	TOTAL DOSE OF BISMUTH	BRAIN WT WET	BISMUTH PER BRAIN	BISMUTH
	mgm	*grams*	*mgm*	*mgm %*
1	600	2.05	0.0335	1.625
2	600	2.10	0.0360	1.715
3	600	2.30	0.0465	2.020
4	600	2.45	0.0280	1.144
5	600	2.55	0.0310	1.215
6	600	2.15	0.0550	2.558
7	600	2.35	0.0520	2.212
8	600	2.15	0.0280	1.320
9	600	2.45	0.0280	1.143
10	600	2.20	0.0250	1.136
11	600	2.35	0.0220	1.004
12	600	2.30	0.0200	0.868
13	600	2.32	0.0200	0.866
14	600	2.28	0.0180	0.789
15	600	2.25	0.0160	0.710
16	500	2.25	0.0180	0.798
17	500	2.30	0.0180	0.795

Since the oral toxicity of dihydroxypropyl bismuthate in rats is so very low, this dosage was chosen completely independent of body weight. It should be kept in mind that according to the previous determination of gastrointestinal absorption estimated from urinary excretion, approximately 2% of the administered bismuth is absorbed. Therefore the total amount of bismuth to enter the circulation hardly could have exceeded 10 to 15 mgm.

Distribution of Bismuth From Chronic Oral Administration of Dihydroxypropyl Bismuthate. An adult, female terrier weighing 6.1 kgm. was given 10 mgm. of bismuth per kgm by stomach tube once daily in the form of dihydroxypropyl bismuthate. At the end of 8 weeks, the dog was sacrificed and the bismuth content was determined in the liver, kidney, brain, spinal cord, blood and cerebral spinal fluid. One kidney was sectioned and examined histologically for pathological changes. The urinary level of bismuth ranged from 2.5 to 4.5 mgm. daily, estimated by intermittent analysis during the ingestion period. Fecal determinations were not done.

A gross examination at the time of sacrifice revealed no bismuth gum line nor other visible effects of the medication. The animal retained a fine appetite and gained weight during the 8 weeks. The histological examination of the kidney was done by a member of the Pathology Department who reported the tissue as essentially normal. This point is of major importance when it is considered that the kidney is the first organ to be damaged by all bismuth preparations. The bismuth content of the organs and fluids analyzed are shown in table 8.

TABLE 8

Distribution of bismuth following oral administration of dihydroxypropyl bismuthate to a dog

TISSUE	TOTAL WEIGHT	AMT TISSUE ANAL	BI. PER ORGAN	MGM. %
	grams	*grams or cc*	*mgm*	
Brain	64.5	64.5	0.260	0.402
Sp. cord		9.0		0.189
Sp. fluid		2.1		0.380
Blood		20.0		0.400
Liver	175.0	15.0	1.165	0.688
Kidneys	29.2	14.6	1.000	3.420

TABLE 9

Recovery of urinary bismuth in university students following a single oral ingestion

SUBJECT	DOSE	URINARY VOL		URINARY BI		% BI. EXCRETED	
		1–24 hr.	24–48 hr	1–24 hr	24–48 hr	1–24 hr.	1–48 hr
	mgm Bi	*cc*	*cc*	*mgm*	*mgm*		
J. B.	50	750	2,100	9.0	2.2	18.0	22.4
E. C.	75	940	780	17.8	1.6	23.8	25.9
R. D.	75	1,600	800	19.2	1.6	25.5	27.8
D. F.	75	1,400	1,680	25.1	1.1	33.5	35.0
R. H.	75	845	1,330	5.6	0.7	7.5	8.0
J. H.	100	1,070	1,380	8.7	0.2	8.7	8.9
C. H.	100	670	670	5.6	0.7	5.6	6.3
G. J.	100	750	1,240	3.0	10.1	3.0	13.1
D. K.	100	980	1,260	13.5	0.2	13.5	13.7
G. M.	100	1,550	1,180	19.4	0.3	19.4	19.7
L. W.	100	900	960	7.5	2.2	7.5	9.7

Human Excretion of Dihydroxypropyl Bismuthate. Twelve normally active university students were selected as subjects to be given oral doses of dihydroxypropyl bismuthate. Total doses of 50, 75 and 100 mgm. bismuth were administered in a solution containing 3% trisodium citrate and the complete 24 hour urine output was collected for two days. No attempt was made to control food or water intake. It will be noted that an average of nearly 15% of the administered bismuth was excreted in the urine. The absorption indicated by such a high excretion is far in excess of any obtained by oral administration in animals.

Daily oral doses were administered to a hospitalized patient, age 21, for approximately 6 weeks The medication was given in capsule form in doses of 50 mgm bismuth once daily The bulk of the capsule contents was comprised of trisodium citrate Excretion under these conditions averaged 3 75 mgm bismuth daily, or approximately 7 5% of the total amount administered The bismuth elimination was accompanied by an increased urine flow, but there was no evidence of an untoward effect There is no explanation at the present time for the difference in the percentage eliminated in the normal active and in the bed rest subjects

DISCUSSION The chemical composition of dihydroxypropyl bismuthate governs its physiological activity in three ways The presence of 2 hydroxyl groups permits complete aqueous and tissue solubility The electronegative, pentavalent form of bismuth insures compatibility with serum proteins as compared to the free metallic ion The weak polarity of such a combination permits stability in a wide pH range

Since gastric juice is one of the barriers presented to soluble bismuth compounds when administered orally, *in vitro* studies were employed to demonstrate that dihydroxypropyl bismuthate is capable of remaining in a soluble form for long periods of time in an acid media Thus normal gastric acidity offers no problem by rendering this compound insoluble

Intramuscularly, the toxicity of dihydroxypropyl bismuthate is in the range of all water soluble bismuth compounds and is governed by the rate of absorption Orally, the toxicity is extremely low as demonstrated in rats In rabbits, a more acute but not alarming oral toxicity is encountered No absorption studies were made in this connection and it is impossible to state whether rabbits are more susceptible to bismuth than rats, or whether the greater toxicity is due to the difference in feeding habits of the two animals

Studying absorption and excretion of dihydroxypropyl bismuthate from oral administration it becomes evident that the chief internal sojourn of the bismuth is in the blood itself So simple a reason can not be offered, nor can any clear explanation be substituted for the absorption of insoluble compounds from intramuscular depots Furthermore that fraction which is not absorbed from the intestinal lining is shortly discharged from the body and no consideration is necessary in connection with permanent presence of innumberable depots releasing subtherapeutic amounts of bismuth The presence of elimination for several days after a single administration gives evidence of small amounts of retention of dihydroxypropyl bismuthate The retention becomes equalized upon chronic administration as indicated by the urinary level of excretion

Chronic oral administration of dihydroxypropyl bismuthate in a dog proved the presence of bismuth in fluids and tissues with absence of kidney damage which is frequently present with this heavy metal Continued oral administration to rats as well as dogs revealed an unusually high concentration of bismuth in the central nervous system Hanzlik Lehman, Richardson and van Winkle (11) using rabbits were unable to demonstrate bismuth in the brains of over 50% of the animals after voluntary drinking of sobisminol The highest brain concentrations obtained were (1) 1 734 mgm % of bismuth following an intake of

14.290 grams of bismuth, and (2) 0.984 mgm. % following an intake of 10.688 grams of bismuth. Using rats voluntarily drinking solution of sobisminol, these investigators were able to obtain an average brain deposition of 0.2 mgm. % after an average consumption of 2.0 grams of bismuth. These reported values are low in comparison to values found in our animals.

The literature reveals no instance where injected bismuth was as effective in cerebral penetration as was the oral route. Hanzlik, Lehman and Richardson (12) offer this direct comparison using sobisminol both orally and intramuscularly. In their study much larger total doses were possible orally than intramuscularly, since the margin of safety intramuscularly was often exceeded.

Experimental oral administration of dihydroxypropyl bismuthate in man shows absorption and excretion of essentially the same amounts of bismuth as proposed by Cole and associates (13) to be indicative of therapeutic activity. Since this compound is well tolerated, has low toxicity and absorption is well within the range of therapeutic activity, we believe it is to be well suited as an oral bismuth medication in the treatment of syphilis.

SUMMARY

(1) The preparation and properties are described of dihydroxypropyl bismuthate, a pentavalent, electronegative bismuth combination, forming neutral aqueous solutions and stable in the fluids of the body.

(2) Toxicity studies reveal it to be relatively nontoxic orally and within range of toxicity established for other water soluble bismuth compounds intramuscularly. Injected intramuscularly in physiological saline, 32 mgm. bismuth is about the minimum lethal dose for rats.

(3) Metabolic studies of continued administration of dihydroxypropyl bismuthate indicates that absorption, distribution and excretion of bismuth results without demonstrable tissue damage.

Clinical studies are being carried out at the present time in the Department of of Dermatology, State University of Iowa Hospitals. The diuretic activity is being studied in the Department of Internal Medicine.

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THE EFFECT OF CHOLESTEROL ADMINISTRATION ON ANESTHESIA

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In the early years of the present century considerable interest was aroused in relationships existing between lipoids and anesthetic agents by the extraordinary findings of Meyer and Overton (6) and their immediate followers. These observations form the basis for the Meyer-Overton theory of the "action" of anesthetic agents. However great the short-comings of this theory are as an explanation of how anesthetic agents produce anesthesia, new data were obtained during these studies which emphasize the interrelationships of lipoid solubility and anesthetic action. In 1936, Starkenstein and Weden published a paper discussing the influence of cholesterol administration upon the activity of hypnotic and anesthetic agents. The purpose of the present study is to repeat in part the experiments of Starkenstein and Weden, and to obtain sufficient data to establish the fact of an additive or potentiating effect of certain lipoids on anesthetic action not only in the case of a volatile lipoid soluble anesthetic agent, ether, but also in the case of a non-volatile agent, a barbiturate (pentobarbital sodium).

MATERIALS AND METHODS. *Animals.* (a) *Mice.* Two hundred four male albino mice were secured from two sources and divided at random into groups of 12. Each individual received a distinctive mark. No food was given 16 to 18 hours before the experimental period.

(b) *Rabbits.* One hundred seventeen rabbits (80% albinos and 20% grays) were secured from several sources and divided into random groups. The sizes of these groups and the sex distribution are shown in the tables. The rabbits were fasted for 16 to 18 hours before the experimental period.

Lipoid and control injections. All substances injected intraperitoneally were warmed to 38°C. before injection. The olive oil used contained less than 0.1% cholesterol; the lecithin (animal) was only 90% pure and contained about 10% cholesterol. The cholesterol emulsions used in these experiments were made according to the method of Cole and his associates (2). The lecithin emulsion was made according to an unpublished method of these workers and was furnished us through their courtesy. The dosages employed were determined in preliminary experiments. These early studies showed that an altered response both to ether and to pentobarbital sodium appeared on repeated injection of the lipoids; accordingly, all animals were used for one experiment only.

(a) *Mice.* In groups of 12, two or three animals at a time were injected intraperitoneally either with 0.25 cc. physiological saline, 0.25 cc. olive oil, 0.25 cc. olive oil containing 5% (12.5 mgm.) cholesterol, 0.4 cc. cholesterol emulsion (12.5 mgm. cholesterol), or 0.4 cc. lecithin emulsion (12.5 mgm. lecithin), respectively.

(b) *Rabbits.* In groups of 6 (usually), one or two animals at a time were injected intraperitoneally either with 4 cc. per kgm. physiological saline, 4 cc. per kgm. olive oil, 4 cc. olive oil containing cholesterol 5% (200 mgm. cholesterol), 6.6 cc. per kgm cholesterol in 3% emulsion (200 mgm. cholesterol) or 6.6 cc. per kgm. lecithin in 3% emulsion (200 mgm. lecithin), respectively.

Anesthesia. (a) *Mice.* Ether. Thirty to 45 minutes following the intraperitoneal injections described above, the animals (12 at a time) were placed in a tight box of 24.5 liter capacity. This had two glass windows. Through a glass tube, one end of which projected into the interior of the box (the other end was closed by a soft rubber tube and a clamp), ether was injected so that it sprayed on a piece of filter paper hanging down from the lid of the box. A sufficient quantity of liquid ether was injected so that on volatilization a vapor concentration of 3.0% by volume was produced. Thirty minutes later the ether vapor concentration was increased to 3.5% and 10 minutes later to 4.5%. These ether concentrations represent values arrived at by calculation on the basis of the total quantity of liquid ether injected. Corrections were not made for the ether absorbed by the animals, since the size and number of the mice were constant from one day to another, and the object of the procedure was merely to obtain an easily reproducible ether concentration. Control and test animals, variously injected, were subjected simultaneously to the given ether concentration during each run.

TABLE 1

The effect in mice of ether inhalation after the intraperitoneal injection of various substances

SUBSTANCE ADMINISTERED INTRAPERITONEALLY	NUMBER OF MICE USED (ALL MALES)	AVERAGE WEIGHT	NUMBER AND PERCENTAGE OF ANIMALS ANESTHETIZED IN A GIVEN TIME IN AN ETHER CONCENTRATION OF						AVERAGE TIME OF RECOVERY FROM ANESTHESIA
			3.0 vol %		3.5 vol %		4.5 vol %		
			No	%	No	%	No	%	
		grams							*minutes*
0.25 cc normal saline	48	24.7	8	16.7	29	67.6	48	100	2.2 ±0.14
0.25 cc olive oil	46	23.8	21	45.7	46	100.0	46	100	4.4 ±0.31
0.25 cc 5% cholesterol in oil	47	23.6	28	59.6	47	100.0	47	100	4.3 ±0.27
0.40 cc of 3% colloidal cholesterol	32	22.6	18	56.4	32	100.0	32	100	4.2 ±0.29
0.40 cc of 3% colloidal lecithin	31	21.8	5	16.1	26	84.0	31	100	2.3 ±0.17

During the anesthesia period, observations were made every five minutes and the condition of the mice observed. Loud tapping with a metal rod on the metal box at 5 minute intervals made it possible to distinguish between sleep and anesthesia. Also the depressed mice received considerable stimulation from the excited active animals running over them. The onset of anesthesia was counted from the time the individual did not change his position in response to the auditory stimulus. At the end of 50 minutes all animals were removed at the same time from the ether atmosphere. The time of recovery as indicated by active movement was recorded for each individual.

(b) *Rabbits.* Ether or Pentobarbital Sodium. Thirty to 45 minutes after the intraperitoneal injections referred to above, the rabbits received 2 cc liquid ether per kgm subcutaneously (nape of neck) or 25 mgm per kgm pentobarbital sodium intravenously. The onset and duration of sleep and anesthesia were recorded. The onset of "sleep" was measured from the time the animals lay on their side until they were awake. The measurement of "sleep" terminated with the first active movement. The onset of anesthesia was measured from the time the righting reflexes were lost until they were recovered. The

TABLE 2

The effect in rabbits of the subcutaneous administration of 2 cc. per kg. ether following the intraperitoneal injection of various substances

SUBSTANCE ADMINISTERED INTRA-PERITONEALLY	NUMBER OF RABBITS (ALL MALES)	AVERAGE WEIGHT	"SLEEP"				ABSENCE OF RIGHTING REFLEX			ABSENCE OF PAIN REFLEX		
			Developed in		After	Duration	Developed in		Duration	Developed in		Duration
			No.	% of total			No.	% of total		No.	% of total	
		kg.			*min.*	*min.*			*min.*			*min.*
4 cc. per kg. of normal saline....	18	2.48	15	84.5	12.4	18.0 ±3.1	12	66.6	12.4 ±2.7	0	0	0
4 cc. per kg. of olive oil	17	2.58	17	100.0	9	29.0 ±2.8	17	100.0	22.5 ±3.0	2	11.8	1.0
200 mg. per kg. of cholesterol in 4 cc.....	18	2.78	18	100.0	10.4	47.6 ±5.2	18	100.0	44.2 ±4.8	9	50.0	6.3

TABLE 3

The effect in rabbits of the intravenous administration of 25 mgm. per kg. of pentobarbital sodium following the intraperitoneal injection of various substances

SUBSTANCE ADMINISTERED INTRA-PERITONEALLY	NUMBER OF RABBITS USED	SEX	AVERAGE WEIGHT	"SLEEP"				ABSENCE OF RIGHTING REFLEX			ABSENCE OF PAIN REFLEX		
				Developed in		After	Duration	Developed in		Duration	Developed in		Duration
				No.	% of total			No	% of total		No.	% of total	
			kg.			*min.*	*min.*			*min.*			*min.*
4 cc. per kg. of normal saline	10	F=7 M=3	2.43	10	100	1.7	43.0 ±5.4	9	90	29.8 ±6.0	1	10.0	3.3
4 cc. per kg. of olive oil	10	F=6 M=4	2.35	10	100	0.7	45.3 ±5.2	10	100	36.6 ±4 2	1	10.0	0.3
200 mgm. per kg. of cholesterol in 4 cc. of olive oil	9	F=5 M=4	2.45	9	100	0	80.5 ±7.4	9	100	77.0 ±6.3	7	78.0	25.6
200 mgm. per kg. of colloidal cholesterol in 3% solution	18	F=9 M=9	2.30	18	100	0.1	80.2 ±3.9	18	100	74.3 ±4.2	15	83.5	30.4
200 mgm. per kg. of colloidal lecithin in 3% solution	17	F=9 M=8	2.38	17	100	1.3	49.0 ±3.9	17	100	28.4 ±3.6	8	43.5	8.1

presence or absence of response to a painful stimulus was tested. After the onset of sleep all animals were regularly stimulated every two minutes by pinching their tails with a hemostat. The state of the corneal reflex was observed.

Results. It is clear (table 1) that the intraperitoneal injection of olive oil, of cholesterol in oil, or of colloidal cholesterol nearly doubles the average time required for recovery from a given ether administration over that observed when the intraperitoneal injection has consisted of physiological saline or of colloidal lecithin. That this prolonged recovery time is significant can be shown by calculation from the standard errors of the means.[1] Outstanding differences in the

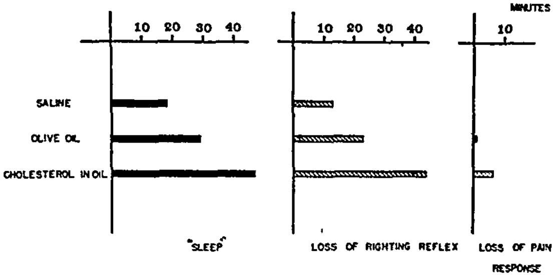

Fig. 1. Rabbits under Ether

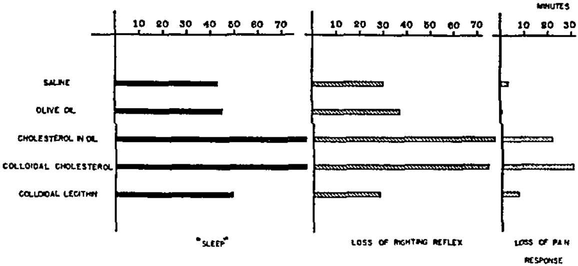

Fig. 2. Rabbits under Pentobarbital Sodium

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[1] The mean recovery time for animals with saline injection was 2.2 minutes (the same also for lecithin) Call this M_1, M_2, M_3, M_4 represent the mean recovery times following olive oil, cholesterol in oil or colloidal cholesterol injection, respectively.

$$M_1 - M_n > 2\sqrt{\overline{SEM}_1^2 + \overline{SEM}_2^2}$$
$$M_1 - M_2 = 2.2 > 2(0.34)$$
$$M_1 - M_3 = 2.1 > 2(0.30)$$
$$M_1 - M_4 = 2.0 > 2(0.32)$$

response of rabbits to the anesthetic agents, depending upon the intraperitoneal injection, are illustrated in tables 2 and 3. In contrast to the effect of cholesterol or oil administration, the intraperitoneal injection of lecithin had no effect either on the development of anesthesia or on the time of recovery.

DISCUSSION. The effect of parenteral cholesterol administration on anesthesia was, as already mentioned, first investigated by Starkenstein and Weden. Though the experimental details given by these authors are fewer than one could wish their major conclusion has been corroborated by our experiments for ether and a barbiturate, namely, that the length and depth of anesthesia produced by various anesthetic agents (they studied ether, chloroform, sodium barbital, urethane, diethylhydantoin) can be greatly increased by previous intraperitoneal injection of cholesterol. We have confirmed their observations for ether and a barbiturate. The difference between their results and ours is chiefly quantitative.

In looking for an explanation of the effect of cholesterol Starkenstein and Weden mention the possibility, without trying to prove it, that its effect lies in the increase of the "transport material" which assists the anesthetic agent in reaching the lipoid-rich tissues of the central nervous system. Our lecithin data do not support this, since lecithin was without effect although it has the same order of ether solubility as cholesterol. It also seems improbable that such an explanation could account for the effect of cholesterol on the barbiturate anesthesia. We wish to emphasize that, judging from these facts, one apparently must look beyond physical solubility and transport effects for an explanation of the cholesterol phenomenon.

At the present time we can give no explanation of the mode of action whereby cholesterol exerts its effect on the anesthetic process. Aird and Gurchot (1932) have presented evidence in an effort to show that cholesterol decreases and lecithin increases the permeability of cell membranes. If this could be shown to be the case it would be of great interest. Much further evidence is required before acceptance of this view is possible.

Evidence of the anesthetic power of sterols is given in the experiments of Selye, who holds that "no compound [steroid] devoid of hormone action possesses this power."[2] While cholesterol appears to potentiate the anesthetic effect of the ether and of the barbiturate, this effect may possibly be only addition. There was some suggestion in the animals studied that the oil or cholesterol administered produced sluggishness of response to stimulation before the anesthetic agents were administered More sensitive methods of studying possible cholesterol or oil depression must be employed before this can be said to be not an addition but a potentiation

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[2] After the conclusion of the series of experiments presented in this paper we learned of a paper published in 1927 by Cashin and Moravek. They report success in inducing anesthesia in cats by the intravenous injection of a watery cholesterol emulsion containing a small amount of lecithin. They do not state the exact dose required for this purpose, but it is evident from their paper that it must have been less than 100 mgm. per kgm., because they state that this dose caused death within 30 seconds and smaller (but unstated) doses produced deep anesthesia lasting for 20 minutes. We also employed intravenous injection of cholesterol emulsions in a number of dogs and rabbits but the majority of animals died shortly after the injection, possibly due to intravascular precipitation of the cholesterol emulsion. If, however, the experiments of Cashin and Moravek can be corroborated, Selye's "law" of steroid hormone anesthesia may require some modification.

CONCLUSIONS

1. We have confirmed for ether and a barbiturate the principal conclusion of Starkenstein and Weden that the depth and duration of anesthesia can be greatly increased by the previous injection of cholesterol

2 The cholesterol effect appears to be a potentiation, the possibility that it may be additive cannot be eliminated at this time

3 In searching for an explanation of the cholesterol action one must look beyond physical solubility and transport effects: (a) Both olive oil and cholesterol increase the effectiveness of ether, but only cholesterol increases the effectiveness of the barbiturate Olive oil has no effect on the barbiturate (b) Ether has the same order of solubility in both cholesterol and lecithin yet the cholesterol increases the anesthetic effect of ether (and the barbiturate) while the lecithin does not

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THE TREATMENT OF STANDARDIZED AND GRADED HISTAMINE SHOCK IN DOGS WITH SOLUTIONS OF METHYL CELLULOSE AND S-METHYLISOTHIOUREA SULFATE

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Previous investigations (1, 2, 3) established the hematic and organic reactions elicited in normal and shocked animals following the single and repeated intravenous injection of several macromolecular plasma substitutes (polyvinyl alcohol, methyl cellulose, pectin, gelatine). Evidence was supplied indicating that methyl cellulose solutions seem to exert a beneficial effect in the treatment of different types of experimental shock in dogs. A method for the development of a standardized and graded histamine shock in dogs was reported, furnishing the basis for a reliable evaluation of the relative efficacy of therapeutic agents used in the management of secondary shock (4).

The present report deals with an extension of these studies on the fundamental dynamics and the therapy of histamine shock by the use of methyl cellulose of different molecular weights and viscosities and by combining this macromolecular colloidal agent with various vasotonic substances, such as *S*-methyl isothiourea, posterior pituitary extract, ephedrine, tyramine, and with detoxicating agents, such as ascorbic acid and calcium glucuronate.

EXPERIMENTAL PROCEDURE. The colloidal agent used in this study was methyl cellulose. This water soluble methyl ether of cellulose was employed in five of the seven available types, which differ from each other in molecular weight, degree of polymerization, and degree of viscosity, but not in colloidal osmotic pressure. The data concerning the molecular weight, degree of polymerization and viscosity were kindly supplied by Dr. R. M. Upright of the Dow Chemical Company, the manufacturer of this product. The molecular weights were calculated from the intrinsic viscosities according to Kramer's modification of Staudinger's formula and represent, therefore, the average molecular weights of these substances (the aggregate sizes of which may occur as larger or smaller molecular components than the average reported). The colloidal osmotic pressures were determined by employing the apparatus and method which involve the use of a cellophane membrane to establish osmotic equilibrium (Simms, Zwemer and Lowenstein). This information is presented in table 1.

The colloidal osmotic pressures were determined for 0.1% aqueous solutions of the respective samples. The viscosity data furnished were based on the viscosities of 2% aqueous solutions as determined in Ubbelohde viscosimeter tubes at 20°C. Additional information on the physicochemical properties of methyl cellulose may be found in previous publications (1, 2). A study of the therapeutic efficacy of colloidal solutions of a substance varying only in molecular size but not in molecular shape or chemical composition seemed to be of special interest, as these factors determine colloidal osmotic pressure and vascular permeability, which are said to be altered in shock.

While the pharmacological and, particularly, vasotonic qualities of posterior pituitary extract and ephedrine are well known and therefore do not need to be discussed here, those of *S*-methyl isothiourea sulfate require some mention, as this substance has only recently been introduced as a therapeutic agent (6, 7, 8). *S*-methyl isothiourea sulfate causes an elevation of the systolic and diastolic blood pressure in man and animals by acting probably through a peripheral mechanism This action is not interfered with by a paralysis of the sympathetic motor effects produced by ergotoxine or *1 3* methyl piperidinobenzodioxane The heart beat is slowed and the respiration is stimulated The vasopressor effects can be elicited by intravenous or oral introduction and are relatively prolonged The substance has been used by Smirk with good results in counteracting experimental shock in animals and traumatic or anesthetic shock (spinal anesthesia) in man No untoward complications have been noted in man and animals after the use of therapeutic doses The solution is acid and does not deteriorate appreciably in this state, but begins to decompose at a pH 9 0 with the liberation of CH_3SH Solutions can be sterilized by tyndallization Some of these observations of Smirk and his collaborators could be confirmed in our own pharmacopathological studies of this substance (9)

Although the use of vasotonic agents in the treatment of shock has at the present time few supporters (10), the rationale of such therapy is evident from the fact that even during shock not the entire arterial system is contracted but only one or several provinces, while the arteries in the other regions are dilated (11) Inasmuch as a prolonged improvement of the circulation and distribution of the blood cannot be expected from a mere increase in blood volume by the introduction of colloidally active liquids into a relaxed and leaking vascular system, a restoration of a proper cardiovascular tonus appears to be a fundamental requirement for a sustained effectiveness of colloidal therapy, as an increased cardiovascular tonus under such conditions not only improves the blood circulation, but also decreases vascular permeability

TABLE 1

Properties of methyl cellulose

VISCOSITY TYPE	MOLECULAR WEIGHT	POLYMERIZATION DEGREE	OSMOTIC PRESSURE, 0 1% SOLN
cps			
15	32,200	169	
25	36,400	191	
50	53,400	281	183
100	60,500	318	183
400	77,700	409	191
1,500	118,200	622	187
4,000	143,600	756	186

The reasoning underlying the use of detoxicating agents was presented in previous publications (2, 12) It was the purpose of the present experiments to gain additional information concerning the efficacy and dosage of some of the members of the mixture employed before

A total of 100 mongrel dogs was used These were injected subcutaneously with histamine dihydrochloride suspended in a base of cottonseed oil (U S P) and "Falba" Absorption Base (Pfaltz and Bauer, Inc) The vehicle was prepared by mixing three parts of the oil with one part of the absorption base (W/W) One cc of the mixture contained 20 mgm of histamine dihydrochloride Injections of the histamine preparation were made after the dogs had been placed under nembutal anesthesia, after the femoral artery had been connected with a mercury manometer for the kymographic recording of the blood pressure, and after the blood pressure had been recorded for 10 to 15 minutes and had become stationary Blood for hematic studies was removed from the jugular vein before the histamine injection and afterwards at more or less regular intervals in many of the animals used The following components of the blood were determined number of erythrocytes and leucocytes, volume of packed blood cells, plasma viscosity, colloid osmotic pressure and erythrocytic sedimentation rate In all instances herein reported the therapeutic agents were administered by intravenous infusion

In the performance of these tests the osmometer, described by Simms, Zwemer and Lowenstein, and the viscosimeter of Hess were used. The various types of methyl cellulose were obtained from the Dow Chemical Company, while the *S*-methyl isothiourea sulfate was the brand of the Eastman Kodak Company. The posterior pituitary extract was prepared in this laboratory.

Animals which survived a primary observation period of seven hours were removed from the operating table, placed into their cages and covered with toweling after the vessels had been ligated, the wounds dusted with sulfathiazole powder and the skin sutured. Autopsies were performed on the majority of the dogs which either died during the experiment or which were sacrificed after surviving the experiment eight to ten days. Histological studies of the internal organs (brain, heart, lung, liver, stomach, intestine, pancreas, spleen, kidney, adrenal, aorta at various levels and carotid artery) were made of a limited number of them so as to ascertain not only the early and late pathologic manifestations of shock, but to search also for any lesions which might be attributable to the treatment given.

1. SHOCK TREATED WITH METHYL CELLULOSE SOLUTION (4000 CPS.) CONTAINING *S*-METHYL ISOTHIOUREA SULFATE. An 0.075% methyl cellulose (4000 cps.) solution in normal saline has a viscosity of 2.0, i.e., that of normal plasma. To this solution sufficient S-methyl isothiourea was added to make a concentration of 1:1000. This particular concentration of *S*-methyl isothiourea was found to be the most suitable for therapeutic purposes after some preliminary experiments with higher and lower concentrations, which gave either too strong and rapid a reaction or an effect which was not intense or lasting enough. The above mentioned water clear, viscous solution, composed of a hydrophilic colloid and a vasotonic agent, has a colloidal osmotic pressure of 134, or approximately equivalent to $\frac{1}{3}$ that of human plasma (300–400 mm. of water) and that of canine plasma (245–365 mm. of water) (13, 14, 15).

The series consists of 42 dogs; 10 were injected with 20 mgm. of histamine dihydrochloride per kilo and 32 received 15 mgm./kg. The blood pressure was lowered thereby to a level of 30 to 45 mm. for a period of 60 to 120 minutes before an injection of 0.075% methyl cellulose plus 0.1% *S*-methyl isothiourea in normal saline was given into a femoral vein. The initial dose of the colloid-vasotonic solution was from 18 to 25 cc. per kg. This treatment was repeated with smaller doses once to thrice during the observation period of 6–7 hours whenever the blood pressure showed a tendency to drop progressively following a primary rise after the first injection. The total amount of fluid intravenously introduced fluctuated between 150 to 500 cc. or from 20 to 50 cc. per kg. The average amount injected was 310 cc., or 29 cc. per kg. The weight of the dogs varied between 6.6 kg. and 18 kg. (average 9.54 kg.).

The types of reaction observed in the dogs shocked with 20 mgm. of histamine dihydrochloride per kg. are illustrated by the two cases presented in table 2, while those shocked with the 15 mgm./kg. dose are given in two examples in table 3.

Colloidal osmotic pressure determinations of the serum were made in nine of the dogs shocked with the 15 mgm./kg. dose. Table 4 presents the data covering this factor in three representative examples of this series.

If the dogs studied are arranged in three groups according to the type of reaction to the injection of the MC + MTU[1] solution: *1*, those in which the treatment did not increase the blood pressure above 60 mm for more than one hour, *2*, those in which the therapy raised the blood pressure to 60–90 mm for one hour but was unable to maintain it at that level, and *3*, those in which the treatment elevated the blood pressure above 100 mm and maintained it there for at least 2 hours, the following tabulation appears

TABLE 2

Reactions to the injection of methyl cellulose S methyl isothiourea sulfate in shock produced by histamine 20 mgm per kg

DOG	WT	TIME*	B.P	E	L	V	TREATMENT	PATHOLOGY
			mm					
1146	9 3	0	120	7 75	10 4	2 15	186 mgm histamine	Heart contracted
		20	30	7 35	1 2	2 00		Lungs congested
		95	34	6 10	1 2	1 90	150 cc MC + MTU†	Spleen large, dark red
		110	62	6 05	2 4	1 90	End of injection	Intestine deep red, velvety mucosa
		180	86	6 60	2 6	2 00		
		240	100	6 85	3 6	2 00		Rectum normal
		300	100	6 45	4 7	2 00		
		360	104	7 45	8 9	2 00	100 cc MC + MTU	
		410	120					Death overnight
1139	9 6	0	112	6 30	24 8	2 90	192 mgm histamine	Heart dilated
		20	32	6 50	4 0	2 40		Lungs congested
		80	42	6 50	4 2	2 35		Liver congested
		120	44				150 cc MC + MTU	Gall bladder edematous
		140	82	8 55	4 2	2 40	End of injection	
		210	66				50 cc MC + MTU	Kidney congested
		225	76	7 45	12 6	2 70		Stomach tarry mucus
		295	60	7 00	13 2	2 70		
		340	0					Duodenum Hemorrhagic mucosa and content
								Ileum spotty hemorrhagic
								Rectum normal

* Minutes after histamine administration

† MC = 0 075% methyl cellulose, MTU = S methyl isothiourea sulfate 0 1 per cent in saline, B P = blood pressure, E = erythrocytes in millions, L = leucocytes in thousands, V = plasma viscosity at 20°C

	GROUP 1		GROUP 2		GROUP 3	
	20 mgm	15 mgm	20 mgm	15 mgm	20 mgm	15 mgm
Cases	4	7	3	12	3	13
Survivals	0	0	0	2	0	9

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[1] Hereafter the following abbreviations will be used MC for methyl cellulose and MTU for *S* methyl isothiourea sulfate

The data recorded on the movements of the plasma viscosity in these dogs indicate that there occurs during the first hours of the shock a mild reduction in the viscosity followed sometimes after injection of the MC + MTU solution by a moderate transitory increase, but usually continuing this decrease for several hours or days when an increase in viscosity occurs, occasionally surpassing the control level. This movement, as shown in subsequent experiments,

TABLE 3

Reactions to the injection of methyl cellulose + S-methyl isothiourea sulfate in shock produced by histamine 15 mgm. per kg.

DOG	WT.	TIME	B.P.	E.	I.	V.	TREATMENT	PATHOLOGY
			mm.	$\times 10^6$	$\times 10^3$			
1168	9.6	0	130	5.85	16.8	2.0	144 mgm. histamine	Organs: normal
		5	32	5.50	1.2	1.8		
		40	34					
		70	46	5.90	2.2	1.85	200 cc. MC + MTU	
		90	90	6.65	2.9	2.0	End of infusion	
		170	116	6.15	3.3	1.95		
		240	126	7.65	9.7	1.95		
		24 hrs.		6.90	25.2	1.95		
		5 days		6.00	13.7	1.98		
		6 days		5.45	23.6	2.0		
		14 days		5.45	11.7	2.9		
1179	12.0	0	104	7.50	14.0	2.6	180 mgm. histamine	Died overnight
		25	50	10.00	2.3	2.6		Heart: dilated
		60	34	9.80	4.7	2.6	200 cc. MC + MTU	Lung: congested, edematous
		75	52	8.90	3.0	2.45	End of infusion	
		165	94	9.60	6.1	2.55		Liver: yellow brown
		260	110					Spleen: cyanotic
		320	110	9.90	10.4	2.95	150 cc. MC + MTU	Duodenum: hemorrhagic
		330	116				End of infusion	Small intestine: spotty hemorrhages Stomach: tarry mucus
1193	9.2	0	102	7.40	4.3	1.85	138 mgm. histamine	Died overnight
		10	22	6.55	0.4	1.7		Heart: contracted
		45	24	8.35	0.2	1.7	200 cc. MC + MTU	Lung: congested
		55	30	6.70	0.3	1.75	End of infusion	Liver: pale yellow brown
		130	38	7.80	0.7	1.9	100 cc. MC + MTU	
		135	36				End of infusion	Spleen: mod. contracted
		155	46	8.00	1.1	2.3		Stomach: Several ulcers in prepyloric region
		195	52				100 cc. MC + MTU	
		215	58	8.25	1.7	2.35	End of infusion	
		300	78				50 cc. MC + MTU	Duodenum: hemorrhagic
		305	82				End of infusion	Ileum: bloody content
		315	74	7.95	7.0	2.51		Cecum: bloody content Rectum: congested

is typical for the effect of MTU, as the injection of MC alone causes a mild to moderate increase in the plasma viscosity during the first twenty-four hours.

The fluctuations of the colloidal osmotic pressure of the serum in the shocked dogs exhibited during the pretreatment period an increase in colloid osmotic pressure, such as seen previously in dogs subjected to histamine shock only (4). This movement was reversed after the injection of the MC + MTU solution. While this response was in general progressive in those animals which died, there followed a return to control or even greater than control values in those which survived. The dogs shocked with histamine and treated unsuccessfully with MC + MTU solution showed, therefore, a movement of the colloidal osmotic pressure opposite from that exhibited by untreated shocked dogs.

TABLE 4

Determinations of the colloidal osmotic pressure of serum

TIME*	COLLOIDAL OSMOTIC PRESSURE			PLASMA VISCOSITY			VOLUME PACKED BLOOD CELLS			BLOOD PRESSURE			TREATMENT		
Dog	1286	1208	1207	1286	1208	1207	1286	1208	1207	1286	1208	1207	1286	1208	1207
0	417	440	407	1 75			52	48	45	112	100	138	Histamine		
10	401	468	487	1 80			56	40	43	42	60	44			
70	466	613	591	1 70			55	46	45	48	34	28	MC + MTU injected		
110	399	518	433	1 80			55	45	43	70	60	46			
170	299	476	430	1 85			56	47	50	66	75	40			
245	534	431	393	1 89			56	49		72	80	30			
290	513	384	388	1 75			57	49		86	90	15			
330	550		352	1 80			55			76		15			
380	453			1 70			57			74					
24 hrs	481			1 55											

* After histamine injection

These results indicate that the ultimate outcome of the shock as influenced by the treatment given depends upon and can be predicted to a certain extent from the response of the blood pressure and colloid osmotic pressure to the therapy. The data provided by hemoconcentration changes proved to be unreliable, as some of the animals which either developed a minor hemoconcentration or even a marked hemodilution died early, while others with a moderate hemoconcentration at the end of the observation period of 7 hours survived. It is remarkable, on the other hand, that 8 of the 19 dogs which exhibited a very satisfactory blood pressure response, sometimes surpassing the control level, died during the 8–18 hour period with the pathological evidences of shock (hemorrhagic duodenum and jejunum, edema of gall bladder, congestion of liver) Whether or not this outcome might have been prevented in some of these dogs if additional infusions could have been given during the night remains uncertain.

The relative efficacy of the treatment administered to the dogs subjected to histamine shock with doses of 20 mgm. and 15 mgm. per kg. is ascertained by a comparison of the lengths of survival time and survival rates of these dogs with those observed previously in dogs which were shocked with the same doses of histamine dihydrochloride and which remained untreated. Table 5 presents these relations.

There is a considerable lengthening of the survival time in both treated series in comparison to the untreated series, and an appreciable increase in the survival rate among the treated animals of the series which received 15 mgm./kg. over that of the corresponding untreated dogs.

An additional series of 12 dogs were then shocked with histamine dihydrochloride (15 mgm./kg.) for testing separately the two constituents of the colloid-vasotonic solution. Seven of these dogs, having an average weight of 8.5 kg., were injected with an 0.075% MC solution in normal saline, whereas the remaining five dogs received intravenous injections of MTU solution (1:1000) in normal saline, given one to two hours after histamine in doses of 20 to 30 cc. per kg. of body weight. The total amount of fluid introduced varied between 150 cc. and 400 cc. (average 290 cc.).

TABLE 5

Survival times and rates of treated and untreated dogs shocked with 15 mgm./kg. and 20 mgm./kg. of histamine dihydrochloride

	DEATH WITHIN			SURVIVALS
	0-7 hrs	*8-18 hrs*	*19-48 hrs*	*per cent*
20 mgm./kg..				
Treated	40	60	0	0
Untreated	80	20	0	0
15 mgm./kg.:				
Treated	15	50	0	33
Untreated	40	50	0	10

The effects obtained with these treatments as reflected by the blood pressure responses and the survival times are presented in the following tabulation.

	BLOOD PRESSURE RATINGS*			SURVIVAL TIME†			
	I	II	III	I	II	III	IV
MC series	2	5	0	1	3	2	1
MTU series	2	2	1	1	4	0	0

* Scheme explained on page 00

† Scheme used in table 5

While the survival time and ratio of dogs treated with MC solution alone is better than that of similarly shocked untreated dogs and dogs injected with MTU solution, it is not as good as in those subjected to injections of MC + MTU solutions.

The two series differed fundamentally in the reaction of the colloid osmotic pressure of the serum. The MC treated dogs, with one exception, exhibited a progressive rise of the colloidal osmotic pressure in the unfavorable cases, similar to that seen in untreated dogs, whereas the dogs of the MTU series showed, with one exception, a moderate to marked decrease of the colloidal osmotic pressure from an average of 482 mm to an average of 283 mm. The exceptional dog in the MC series, which survived for 30 hours, developed first a transitory drop with a subsequent steep rise in colloidal osmotic pressure shortly before death In the exception in the MTU series the colloidal osmotic pressure first underwent a rapid reduction followed by a gradual rise to approximately the normal level reached at the six hour mark.

The plasma viscosity in the MC series showed a tendency toward a mild rise, *while that of the MTU series dropped slightly, except for one dog which displayed* a rapid and marked increase in the number of erythrocytes, leucocytes and viscosity of the plasma during an 18 hour period.

The autopsy findings of these dogs were typical for shock.

2 SHOCK TREATED WITH METHYL CELLULOSES (VISCOSITY 1500 TO 50 CPS.) PLUS *S*-METHYL ISOTHIOUREA SOLUTION. In an additional series of 19 dogs shocked with various doses of histamine dihydrochloride, methyl cellulose solutions prepared from methyl celluloses of lower molecular weights and viscosities (3 dogs with methyl cellulose, 1500 cps., 10 dogs with methyl cellulose, 400 cps , 3 dogs with methyl cellulose, 100 cps , 3 dogs with methyl cellulose, 50 cps) were used. Difficulties were encountered in the preparation of these solutions in connection with the methyl celluloses 1500 cps , 100 cps. and 50 cps., as these types did not give an entirely water clear solution even after filtration through "Hy-flo Supercel" and filter paper. All solutions were adjusted to a viscosity of 2 and therefore varied in concentration (MC 1500 cps , 0.17%; MC 400 cps , 0 25%; MC 100 cps., 0 25%; MC 50 cps , 0.5%). The MTU was added to this *solution in concentrations of 1:1000 to 1:100. The colloid-vasotonic solutions* were administered 60 to 120 minutes after the injection of the histamine in amounts similar to those employed in the preceding experiments.

The results obtained and observations made are summarized in the following tabulation.

TYPE OF MC CPS	NUMBER OF DOGS	DOSE OF HISTAMINE	BLOOD PRESSURE RATINGS			SURVIVAL TIMES			
			I	II	III	I	II	III	IV
		mgm							
1,500	3	5	1		2	1	1		1
400	10	5 to 20		4	6	1 + 7*			2
100	3								
		10	1	2	3	2	1		3
50	3								

* Killed at end of 7 hours by intravenous injection of formalin

The blood pressure responses as well as the survival times of these dogs indicate a relatively favorable reaction to the treatments given This impression receives additional support from the fact that in the MC-400 cps. series increased erythrocytic and hematocrit values dropped after the injection of the colloidal-vasotonic agent to approximately control conditions and prevailed at this level for some time after the treatment. There was only one exception in which a progressive hemoconcentration occurred. Moreover, three of the eight dogs of this series which were killed or died during the first seven hours showed gross evidence of shock. While it is likely that some of the animals might have died later with such changes, there is good reason to believe that an appreciable number of those with good blood pressure responses and favorable hematic reactions would have survived. The survival rate of 20 per cent of this group is therefore presumably much higher than apparent from the actual figures.

The plasma viscosity showed in these dogs a slight rise during the first 7 hours.

3. SHOCK TREATED WITH METHYL CELLULOSE SOLUTION PLUS ADDITIONS OF VARIOUS VASOTONIC AGENTS (POSTERIOR PITUITARY EXTRACT, EPHEDRINE SULFATE, AND TYRAMINE HYDROCHLORIDE). Additional vasotonic agents, such as posterior pituitary extract, ephedrine sulfate and tyramine hydrochloride, were added in place of *S*-methyl isothiourea sulfate for comparative purposes. The incorporation of these agents into the colloidal medium seemed to offer the advantages of a prolonged introduction of the vasotonic substances, with the accompanying less acute effect upon the blood pressure possibly accentuated by a slower absorption from its viscous vehicle, thereby obtaining a more gradual and persistent action.

Ten dogs shocked with histamine dihydrochloride (10 mgm. to 40 mgm./kg.) received intravenous injections of MC (400 cps.) solutions (1% to 0.25%) to which posterior pituitary extract (surgical strength) was added in concentrations of 1:500 to 1:7000. Four additional dogs shocked by similar doses of histamine dihydrochloride were treated with a 0.25 per cent MC solution (100 cps.) containing ephedrine sulfate in a concentration of 1:500 to 1:1000. A second group of four dogs shocked with histamine dihydrochloride (10 mgm. to 20 mgm./kg.) were injected with a MC solution (100 cps.) to which tyramine hydrochloride (1:500 to 1:1000) was added.

The comparatively fleeting marked rise of the blood pressure with these drugs necessitated making the injections at relatively frequent intervals so as to maintain the blood pressure at a moderately high level. Many of the dogs received 6 to 9 injections and thus rather large amounts of fluid (up to 700 cc.). The efficacy of the various treatments is apparent from the following tabulation:

TYPE OF TREATMENT	NUMBER OF DOGS	BLOOD PRESSURE RATINGS			SURVIVAL TIMES			
		I	II	III	I	II	III	IV
MC (400 cps.) + posterior pituitary...	10	2	5	3	2	+8*		
MC (100 cps.) + ephedrine or tyramine...	8	6	2		1	6		1

* Killed at end of observation period of 7 hours.

The fleeting action of posterior pituitary extract upon the blood pressure of dogs shocked with histamine dihydrochloride (15 mgm./kg.) was demonstrated in a second group of four dogs which received the posterior pituitary extract in normal saline solution. In only one instance did this treatment result in a pressor response which was maintained above 100 mm. for a period of several hours.

4. SHOCK TREATED WITH METHYL CELLULOSE (400 CPS.) AND ASCORBIC ACID AND CALCIUM GLUCURONATE SOLUTIONS IN CONJUNCTION WITH METHYL CELLULOSE (400 CPS.) + *S*-METHYL ISOTHIOUREA SOLUTION. In an experiment comprising eight dogs, the treatment of shock with an 0.25 per cent MC (400 cps.) + MTU solution (1:1000) was supplemented by freshly prepared and neutralized solutions of ascorbic acid and calcium glucuronate given separately or mixed with the MC + MTU solution. Four dogs received calcium glucuronate either alone (2) or in combination with ascorbic acid. The calcium glucuronate solution was administered as a 20% solution in quantities of 10 to 50 cc. The shocking dose varied from 5 mgm./kg. to 15 mgm./kg. of histamine dihydrochloride. The calcium glucuronate solution alone had no appreciable effect upon the blood pressure. Inasmuch as it exhibited a marked tendency to form brown floccules soon after its preparation, this substance can not be used with any advantage in the treatment of shock without additions which stabilize the solution.

In a second set of four dogs ascorbic acid in single doses of 5 to 20 gms. in a 20 per cent neutralized solution were used in conjunction with the MC + MTU treatment. The dogs were shocked with histamine dihydrochloride (5 mgm./kg.) and the treatments were begun 45 to 190 minutes after the introduction of the histamine. In two dogs this procedure was repeated three times with intervals of three days between the individual shock episodes, while the shock dose was increased in one dog to 10 mgm./kg. No effect upon the blood pressure was noted in those instances in which the ascorbic acid solution was given apart from the MC + MTU. It was observed, on the other hand, that a too rapid injection of such concentrated ascorbic acid solutions can produce a rapid and severe drop in blood pressure which may simulate that produced by histamine. When mixed with the MC + MTU solution ascorbic acid did not interfere with the usual pressor response, which in most instances was good, carrying and maintaining the pressure beyond the control level and above 100 mm. The observations made with this treatment do not permit a definite statement as to its merits. However, it is apparent that the intravenous introduction of large amounts of ascorbic acid did not do any harm and may have done some good, as suggested by the survival of two of the dogs through three successive episodes of shock sustained in intervals of two to three days.

COMMENT. The evidence presented indicates that dogs subjected to a standardized and graded histamine shock are distinctly benefited by the intravenous injection of a colloidal solution of the hydrophilic MC having a viscosity of two and containing MTU in a concentration of 1:1000 serving as a vasotonic agent.

Animals treated with this solution one to two hours after the institution of the shock showed a considerable lengthening of the survival time and an appreciable increase of the survival rate. The absolute results depend on the severity of the shock produced, i.e., the dose of histamine dihydrochloride given.

These effects most strikingly demonstrated in dogs treated with solutions containing MC (4000 cps.) were apparent also to a somewhat less marked degree in experiments in which the methyl celluloses of lower viscosity were used, especially that of 400 cps. viscosity. The extent of the protection supplied by this type of treatment is illustrated by the fact that only one out of ten untreated dogs shocked with 15 mgm./kg. of histamine dihydrochloride survived, while one out of three of the series treated with a solution of 0.075% MC (4000 cps.) plus MTU (1:1000) in normal saline survived. These favorable results were obtained only when both a hydrophilic colloid and a vasotonic agent were administered together. Either one alone was definitely less effective, especially the vasotonic agent. This combination of the two agents apparently provides the needed amount of fluid and osmotically active hydrophilic colloid of large molecular size, gradually increases the vascular tonus over prolonged periods and counteracts the marked elevation of the colloidal osmotic pressure of the blood serum occurring in shock, without unduly increasing the viscosity of the plasma. The treatment of histamine shock with MC + MTU solutions was ineffective in all those dogs which showed within the first 7 hours of the experiment bloody discharges from the bowel, thereby indicating an irreparably damaged capillary system.

Of the methyl celluloses used in these studies, the one with the highest molecular weight (type 4000 cps., molecular weight 143,000) seemed to be superior to the others, not only in regard to the results obtained but also for the following reasons: Its solution is easily prepared and is water-clear without filtration; it is more stable after autoclaving than those of the other types which do not give entirely clear solutions even after filtration; a much smaller amount of methyl cellulose (4000 cps.) is required to give a solution of viscosity 2 than when the other types are used; solutions of methyl cellulose (4000 cps.) seem to slow and prolong the vasotonic action of MTU to a higher degree than those of the other types; the hydremic effect of MC (4000 cps.) and thus its water-binding power is more persistent than that of the 400 cps. type, as animals injected with this latter material showed already during the first eight hours a mild to moderate increase in the viscosity of their plasma, thus indicating an escape of part of the fluid and a retention of the viscous colloid in the blood.

It is evident from these data that the beneficial effects exerted by the colloid do not depend to any significant degree upon its colloidal osmotic activities, that is, upon its ability to withdraw water from the tissues into the blood. The drop of colloid osmotic pressure of the serum in nephrotic patients following the injection of acacia solution observed by Goudsmit, Binger and Keith (16) as well as the hemoconcentration found by Harkins (17) in dogs subsequent to the intravenous introduction of concentrated serum are observations supporting this conception.

Additional confirmation seems to be supplied by the finding of Mahoney, Kingsley and Howland (18), who noted that half diluted plasma was more effective than normal plasma and that concentrated plasma was least efficient in maintaining life of shocked dogs. It is obvious, moreover, that the influx of tissue fluids into the blood after severe hemorrhage is not the result of an increased plasma protein content of the blood, as this protein content remains either normal or becomes reduced after hemorrhage. Beattie and Collard (19) recorded that after hemorrhage the restoration of blood volume is independent of the restoration of the plasma proteins, as the speed of the blood volume restoration outstrips the rate of plasma protein movement. The observations of Beattie (20), and of Sharpey-Schafer and Wallace (21) on the retention of injected serum or plasma in the circulation and on the movement of plasma, plasma proteins and tissue fluids with their proteins from and into the blood have shown that massive movements of these liquids and their constituents may occur in a relatively short time and that much is unknown concerning the mechanisms controlling the plasma volume and mass movements of plasma proteins. The validity of this statement is strikingly illustrated by the fact that after shock by local crush injury followed by local extravasation of blood fluid into the injured area there occurs, according to the observations of Blalock and his coworkers (10), not a restitution of the lost blood volume by an influx of tissue fluid into the blood from the intact regions of the body with presumably normally functioning vessels, such as seen after hemorrhage, but there develops a progressive hemoconcentration. Our own investigations have shown, moreover, that in histamine shock the colloid osmotic pressure of the serum is not decreased but is appreciably increased. These observations militate against the correctness of the conceptions underlying the presently widely used colloid therapy of shock.

It is essential, when drawing conclusions from the colloid osmotic pressure of plasma and its various substitutes, that consideration be given to the fact that no direct relations exist between colloid osmotic pressure and the molecular size of a particular colloidal agent. Membrane permeability of macromolecules not only depends upon the weight of the molecule, but also upon its configuration (symmetrical, asymmetrical, globular, filamentary), its water binding power (hydrated shape and size), its state of association (molecular or micellar), its shape in solution (stretched or unstretched), the pore size of the membrane, and the presence of detergents (Meyer). Meyer noted that methyl cellulose is conceivably stretched when associated to form micells and resembles polypeptides in this respect The globular shape of the serum albumins and globulins with their relatively large diameter in relation to their molecular weight contrasts sharply, therefore, with the filamentary molecules of gelatine, fibrinogen, methyl cellulose, polyvinyl alcohol and pectin, which possess a comparatively small diameter in regard to their molecular weight. It is noteworthy moreover that the same factors influence the viscosity of their solutions, as solutions of globular molecules have a much lower viscosity than those of filamentary molecules.

In previous studies attention was called to the pathological lesions observed in the hearts and arteries of shocked animals. The histological investigations made of the tissues of the present series confirmed and extended these findings (myocardial degenerations and calcifications, fibroblastic scars in myocardium, medial degenerations and intimal thickening in aorta) which will be reported elsewhere in detail (22). The pathological and symptomatic observations made in these studies furnish a sound basis for the vasotonic therapy advocated. These experiments have shown that among the various vasotonic agents tried (*S*-methyl isothiurea sulfate, posterior pituitary extract, ephedrine sulfate, tyramine) *S*-methyl isothiourea had the most prolonged and consistent effect. A cardiovasotonic therapy in conjunction with a colloidal one appears to be a rational procedure in the management of shock.

The histological examination of the organs of dogs shocked with histamine dihydrochloride and treated with methyl cellulose and *S*-methyl isothiurea solution, which died during the 8 to 18 hour period or were sacrificed 8 days after the shock, showed a complete absence of any storage phenomena or any other lesions indicative of organic injury attributable to the therapeutic agents used. All changes observed (gastric ulcers, hepatic congestion, necroses and interstitial brosis, myocardial degeneration, hemorrhages, calcifications and fibrous scars, odenal hyperemia and hemorrhages, necrosis and edema of gall bladder, splenic hemorrhagic infarctions) were caused by the circulatory disturbances, the associated anoxemia and the reparatory manifestations associated with the shock.

CONCLUSIONS

Dogs shocked with graded doses of histamine dihydrochloride suspended in an oil-Falba vehicle and treated with solutions of methyl cellulose of different molecular weights combined with *S*-methyl isothiourea sulfate showed an appreciable lengthening of the survival time and the survival rate.

These effects were most striking when a 0.075% methyl cellulose (4000 cps.) solution containing *S*-methyl isothiourea sulfate (1:1000) was used.

The combination of the colloidal agent with the vasotonic gave considerably better results than each agent used separately.

Among the various vasotonic agents tested (*S*-methyl isothiourea sulfate, posterior pituitary extract, ephedrine sulfate, tyramine) the *S*-methyl isothiourea gave the most prolonged vasopressor reaction, which was less acute but more persistent when the *S*-methyl isothiourea was administered in the viscous, colloidal methyl cellulose solution.

The various hematic reactions studied (plasma viscosity, colloid osmotic pressure of serum, packed blood cell volume, number of erythrocytes) indicate that the favorable effect of the treatment used is not related essentially to the colloid osmotic pressure of the liquid introduced.

The evidence advanced shows that the therapy used does not cause any demonstrable ill effects attributable to the physico-chemical properties of the colloidal and vasotonic agents.

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NARCOSIS INDUCED BY CARBON DIOXIDE AT LOW ENVIRONMENTAL TEMPERATURES[1]

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The total oxygen consumption of the albino rat is reduced significantly when this animal is placed suddenly in an atmosphere containing 10% CO_2 (1). It is difficult to determine by observation alone whether or not a parallel decrease in the total body activity of the animal occurs since the hyperpnea is so marked as to obscure any manifestations of depression. In normal man, unconsciousness usually occurs at this level of CO_2 in spite of the dyspnea, indicating that some structures within the nervous system are depressed. Since it is of some theoretical, as well as practical interest to know whether or not this and lesser concentrations of carbon dioxide exert a depressant action, the following experiments have been undertaken. They have been based on the fact that even minor grades of depression reduce the capacity of an animal to adjust itself to a cold environment (2). The results obtained, even with the lower concentrations of CO_2, were so striking that parallel experiments have been made with other agents in order to determine, if possible, the relation of the phenomenon to total oxidative metabolism.

METHOD. The experiments were conducted in a 700-liter insulated chamber which could be maintained at the desired temperature by operation of the contained refrigeration unit. The air in the chamber was agitated by the fan of this unit. The chamber was connected in series with a 100-liter spirometer, a 200-liter tank (to increase volume of system), and a 200-liter animal chamber (maintained at room temperature). Circulation throughout the whole closed system was maintained by means of a small blower. A third animal chamber of 100 liter capacity was constructed of glass and placed within the large cold chamber. This third chamber contained a thermostatically controlled heating element, and the gas circulating from the remainder of the system was passed through this chamber before entering the cold chamber. It was possible, therefore, to expose different groups of animals to the identical CO_2-containing atmosphere at room temperature and at two other temperatures simultaneously.

The experiments at high O_2 tensions were carried out in a small bomb placed in the cold chamber and connected to an oxygen cylinder. Carbon dioxide was removed by soda lime so that the concentration in the bomb never exceeded 0.3%.

Rats, rabbits and dogs served as the experimental animals. The rats were placed in small individual wire cages with wooden floors to avoid excessive heat loss by contact with metal. The cages were small enough to restrict activity but not to cause excessive heat loss by conduction through the wire screen. The rabbits and dogs were placed directly in the large chamber with shavings covering the floor.

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RESULTS *A Narcosis induced by CO_2 and cold* When animals are placed in a suitable CO_2 containing atmosphere at an environmental temperature of 5°C , narcosis is produced The time required to produce the state of narcosis varies with the concentration of the gas, the species, and previous condition of the animal used As shown in table 1, 11% CO_2 at an environmental temperature of 5°C appeared to be the optimum concentration for the production of narcosis in the albino rat, since the induction time was convenient and the rat showed no ill effects when exposed to this concentration of CO_2 at room temperature (1) During the first two hours in such an environment the rat exhibits the usual respiratory response to CO_2 and polyuria occurs, but no other gross changes are observed Generalized shivering and depression are then noted and a fall in body temperature occurs As the body temperature decreases, the pulse and respiration also decrease The following events occur in sequence within 6 hours at the indicated body temperatures cessation of shivering, cessation of voluntary movement at 23°C , disappearance of corneal and wink reflexes at 16–17°C , and finally death at approximately 14°C During narcosis (body temperature 16–20°C), the heart rate is between 30 and 100 per minute and the electrocardiogram shows a reduction in the P wave and A V block changes that are also characteristic of hibernating animals The respiratory rate is between 1 and 20 per minute No cyanosis is observed at any time

TABLE 1

Effect of various concentrations of CO_2 on the production of narcosis in the albino rat at 5°C

CONCENTRATION	NUMBER OF ANIMALS	INDUCTION TIME	COMMENT
		hours	
18% and above	10	<2	100% narcosis
15%	5	2 3	100% narcosis
11%	100	3–6	Occasional resistant animal
5%	12	12 20	One resistant up to 48 hours
0%	15	13–25	Three resistant up to 48 hours

After narcosis is induced it is necessary to raise the environmental temperature from 5°C until it approaches the experimental body temperature if recovery of the animal is expected When the rat is exposed to 11% CO_2, the critical environmental temperature for the prolongation of this state appears to be 21°C Above this temperature the rate of heat production exceeds that of heat loss the rat gradually regaining a normal body temperature and activity When the rat is allowed to breathe room air during narcosis the critical environmental temperature for maintenance of this state is 19°C In either 11% CO or air, an environmental temperature below 14°C produces a progressive fall in body temperature during narcosis, and death occurs in a few hours Rats may be maintained in the narcotized condition up to 24 hours if the environmental temperature is kept between 18°C and 20°C [3] However, since states of lowered body temperature are associated with pulmonary edema (5), respiration fails gradually, and death occurs in most animals which have been narcotized longer than 15 hours.[4]

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[3] If the state of narcosis is maintained for several hours a marked opacity of the eyes is noted This state is completely reversible, disappearing as the body temperature returns

When the narcotized animals are removed to air at room temperature no convulsions are seen. Ninety-five per cent recover within 2 to 3 hours after 4 to 5 hours of narcosis, but the incidence of survival decreases markedly if narcosis is prolonged.

The acid-base balance during narcosis was determined on 3 rats which were removed from the large cold chamber as soon as narcosis was induced by 11% CO_2 and placed immediately in the small animal chamber at a temperature of 20°C. where they were exposed to the same atmosphere. Two hours later arterial blood samples were taken from the aorta and analyzed by the method of Shock and Hastings (6) for pH and CO_2 content. At the temperature of the animal (20°C.), the pH of the blood was found to be 7.04 (range, 7.00–7.07), and the CO_2 content 110 vols. % (range, 107–114). The total CO_2 content checked well with values previously reported for the fourth hour of acute exposure to 11% CO_2 at room temperature, but the pH was lowered, probably due to the increased solubility of H_2CO_3 at the lowered body temperature.

A similar type of narcosis may be produced in the rabbit by concentrations of 18% CO_2, or above, at an environmental temperature of 5°C. Reflexes disappear before a body temperature of 25°C. is reached, and at a temperature of 19°C. the respiration is 3 to 22 per minute and the heart rate approximately 30 per minute. Upon removal to air at room temperature, practically all rabbits develop tetanic convulsions which may be relieved by CO_2 administration. Survival following this procedure is rare since the high concentration of CO_2 necessary to produce narcosis in this animal causes extensive pulmonary edema (1).

In the dog, a concentration of 16% CO_2 at 5°C. will produce narcosis in 26 hours. At a body temperature of 19°C., reflexes are abolished, the respiration is 5 per minute and the heart rate 10 per minute, with a femoral pulsus trigeminus. Upon removal to room atmosphere and temperature, tetanic convulsions occur which are relieved by CO_2 administration. The body temperature returns to normal in about 24 hours and recovery occurs. Tarry stools are noted, indicating congestion of, and hemorrhage from, the gastrointestinal tract.

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to normal. Histological examination indicates that this opacity is located in the lens capsule. We have observed it also in states of lowered body temperature produced by cold and high oxygen and by morphine and cold, and Selye (3) states that it may result from any severe injury to the organism. In this connection it is of interest that tum Suden (4) reports that such subcapsular lens opacities may be produced by toxic doses of adrenaline and increased by histamine and that they "appear to be related to some aspect of carbohydrate disturbance . . . and the concomitant circulatory impairment."

[4] An incidental objective of the present study has been to induce hibernation in non-hibernating animals. If the pathological changes in the membranes of the eye and the lung serve as an index of similar changes which are occurring generally throughout the body during exposure to cold, it seems improbable that this objective will be attained.

B Factors modifying the production of narcosis

1 *Controls* In a previous paper (1) we have reported that at ordinary temperatures albino rats may be exposed to 11% CO_2 for at least 30 days with few untoward effects

The data in table 1 indicate that under the conditions of these experiments, in which movement of the animal is restricted by the small cage, 25% of the rats are narcotized at a temperature of 5°C , even when breathing air, although it requires longer than 12 hours of exposure to induce this state Rats allowed full freedom of movement are unaffected by 7 days of exposure to a temperature of 5°C (See also (7)) Therefore, under ordinary conditions neither the concentration of CO_2 used here nor the temperature employed is alone sufficient to induce narcosis

TABLE 2

Factors affecting the induction of narcosis in the albino rat with 11% CO_2 at 5°C

PREVIOUS CONDITION	NUMBER OF ANIMALS	INDUCTION TIME IN 11% CO_2 AT 5°C	COMMENT
		hours	
Normal males	10	3-6	5% mortality
Normal females	10	3-6	
Young rats (150 gms)	10	3-6	
Old rats (300 gms)	10	3-6	
Dehydration	5	3-6	
Starvation	40	2-4	50% mortality
3 weeks in 10% O_2	18	2 3	
16 hrs in 11% CO_2	5	3-6	
24 hrs in 11 20% CO_2	10		No narcosis
Repeated narcotizations	30	6-12	Some completely resistant
7 days at 5°C	5		No narcosis
Thyroid administration	6		No narcosis
Dinitrophenol administration	4	3-4	No significant change

2 *Concentration of CO* The effect of varying the concentration of CO_2 on the time necessary to induce narcosis at a temperature of 5°C is shown in table 1 It is evident from these studies that all concentrations of CO_2 above 5% have certain narcotic properties It is quite clear that either the temperature regulatory mechanism is rendered inactive or heat production is reduced or both, since 5% CO at 5°C produces narcosis in 90% of the animals while cold alone produces narcosis in only 25 per cent of the animals under the conditions of these experiments

3 *Previous treatment or condition of the rat* Certain factors which influence the production of narcosis are presented in table 2 Weight, sex, and previous

dehydration have no appreciable modifying effect, the induction time varying between 3 and 6 hours in all cases. This is considered to be the normal time range for the production of narcosis by 11% CO_2 at 5°C.

Fasting or previous exposure to 10% O_2 increase the susceptibility of the rat to the narcotic action of 11% CO_2 at 5°C. Withholding food for 24 hours before exposure decreases the induction time to 2 to 4 hours and increases the mortality from 5% to nearly 50%. A similar increase in susceptibility is observed in rats which have lost weight steadily during three weeks of exposure to 10% O_2. In this state, as in acute starvation, glycogen stores are reduced and a metabolic acidosis exists.

Previous exposure to 11% CO_2 for 16 hours or less before lowering the environmental temperature to 5°C. does not affect the onset of narcosis. On the other hand, rats which have been acclimated to 11 to 20% CO_2 at room temperature for 24 hours or longer, do not become narcotized when they are subsequently exposed to 11 or even to 20 per cent CO_2 at 5°C. Repeated narcotization with CO_2 at 5°C. at intervals of 4 to 10 days increases the induction time from 3–6 hours to 6–12 hours, and a few rats become completely resistant. The resistance to narcosis developed by either of the above methods disappears fairly rapidly and is completely absent within 2 to 3 weeks.

Increasing the basal metabolism of the rat confers immunity to the narcotic action of 11% CO_2 at 5°C. Exposure of the rat to a temperature of 5°C. in air for a week will increase the metabolic rate (7, 8, 9, 10). Such exposure completely prevents the production of narcosis when 11 to 20% CO_2 is subsequently applied at 5°C. Administration of desiccated thyroid in doses of 1 gram per kgm. orally on three successive days increases the oxygen consumption 20 to 45% by the third day following the last dose and exposure to 11% CO_2 at 5°C. at this time produces no narcosis in 24 hours. Smaller increases in metabolism prolong the induction period up to 36 hours but the animals finally become narcotized.

The administration of 30 mgm./kgm. of dinitrophenol intragastrically does not affect the induction time, although, according to Tainter (11), this dose is sufficient to cause a 100% increase in the oxygen consumption in rats at 25°C These results might be expected in view of Tainter's observation that this dru causes a slight decrease rather than an increase in metabolism at low environmental temperatures.

C. Narcosis with low oxygen tensions and cold. Since Gellhorn (12) has note a fall in body temperature in mice exposed to atmospheres containing 10% oxygen, and a greater fall when 3% CO_2 is added, the effect of low oxygen tensions on the production of narcosis by CO_2 and cold was studied. The result are shown in table 3. It will be seen that whereas atmospheres containing 10% O_2 and 90% nitrogen at an environmental temperature of 5°C. will produce narcosis in 29 to 32 hours, the addition of 5% CO_2 will reduce the induction time t from 7 to 11 hours. Five per cent CO_2 in air at 5°C. will produce narcosis in 12 to 20 hours. Thus, an increase in the CO_2 concentration from 0 to 5% is a mor effective means of inducing narcosis at 5°C. than a reduction in the O_2 concentration from 21 to 10% Moreover, the type of narcosis produced by low oxygen *tensions at 5°C is dissimilar to that induced by CO_2 and cold* As the body temperature falls the rats lose their sense of equilibrium and move with a peculiar rolling motion associated with clonus Movements persist until the body temperature falls below 20°C, and death occurs before a body temperature of 16°C is reached

D Narcosis with high oxygen tensions and cold Campbell reports that at high tensions of oxygen there is a fall in body temperature (13) and a marked increase in the CO_2 content of the tissues (14), the concentration of CO_2 in tissues being as high as 30% at four atmospheres oxygen pressure Bean and Bohr (15) demonstrated that the effects of high oxygen tensions on smooth muscle were similar to those produced by low oxygen tensions and by cyanide, and Bean (16) showed that high oxygen tensions decrease oxygen consumption The effect of high oxygen tensions on narcosis was therefore tried, and the results are shown in table 3 It will be seen that oxygen under four atmospheres pressure at a temperature of 5°C will produce narcosis in 6 hours The narcosis resembles that produced by low oxygen tensions and cold, except that tetanic convulsions occur on removal Clouding of the eyes is noted, the cloudiness disappearing 4 hours after removal Tetanic contractures of all skeletal muscles initiated by handling or other stimulation occur for 24 hours following removal *Death occurs from pulmonary congestion One and two atmospheres of oxygen* at 5°C are not effective in 48 hours

TABLE 3

The incidence of narcosis in the albino rat induced by low and high oxygen tensions at 5°C

GAS CONCENTRATIONS	NUMBER OF ANIMALS	NUMBER NARCOTIZED WITHIN 48 HOURS	INDUCTION TIME
			hours
21% oxygen (air)	15	3	13 25
21% O_2, 5% CO_2	12	11	12 20
10% O_2, 5% CO_2	5	5	7-11
10% O_2, 90% N_2	10	9	29-32
400% O_2	3	3	6
100-200% O_2	2	0	

DISCUSSION From the above experiments it is evident that all concentrations of CO_2 above the physiologic level interfere with the capacity of an animal to adapt itself to a cold environment when such concentrations of CO_2 are applied acutely, but that even 20% CO_2 may be tolerated by the rat after 24 hours of exposure to CO_2 These results may be correlated with the effect of CO_2 on oxidations since, as shown in a previous paper, oxygen utilization is reduced during the first 6 to 8 hours of exposure but returns to normal within 24 hours Further evidence that the interference with temperature regulation by CO_2 is probably due to a general decrease in oxidations and not solely to a central depression *of the temperature regulatory centers is afforded by the protection* conferred when the metabolism is increased by exposure to cold or by thyroid administration. Apparently, when the level of metabolism is raised sufficiently, the decrease in oxidations induced by acute exposure to CO_2 is not great enough to lower the heat production to the point where it is exceeded by the heat loss, and thus no lowering of body temperature with resulting narcosis occurs. The fact that a somewhat similar although not identical state may be induced by decreasing the available or utilizable oxygen by exposure to low or high tensions of oxygen is consistent with this explanation.

A comparison of the effects produced by CO_2 and diminished oxygen at low environmental temperatures indicates that all concentrations of CO_2 above the physiologic level have a depressant action on the central nervous system when applied acutely. In narcosis induced by low oxygen and cold, voluntary movements persist until the body temperature falls below 20°C., or nearly to the temperature at which cold alone is sufficient to maintain narcosis. However, during narcosis induced by CO_2 and cold, voluntary movement ceases before a body temperature of 23°C. is reached, indicating additional depression of the animal by the CO_2.

The narcotic state produced by CO_2 and cold resembles in certain respects both anesthesia and hibernation. The bradycardia, cardiac rhythm, bradypnea, hypothermia and low pH are similar to those found in true hibernation, and the complete relaxation and absence of reflexes are similar to those in anesthesia. This narcotic state differs from hibernation in that the animal can not be aroused by stimulation and death occurs if the body temperature is reduced below a clearly defined level which is considerably above that tolerated by hibernating animals.

SUMMARY

A reversible state of narcosis having certain characteristics of both hibernation and anesthesia may be induced and maintained for many hours in the rat and dog by sudden exposure at 5°C. to concentrations of CO_2 of 5 per cent or greater. In this state the rat has a body temperature of 16–20°C., a heart rate of 30–100 per minute, and a respiratory rate of 1–20 per minute. When the rat is respiring 11% CO_2 at 5°C. the CO_2 content of the blood may reach 110 volumes per cent and the plasma pH a level of 7.07. A similar state may be induced in the rabbit by 20% CO_2 and cold but it is not reversible because of the fatal pulmonary edema.

Rats are rendered more susceptible to this state by fasting, prolonged (three weeks) exposure to 10% O_2, or by administration of small doses of depressant drugs.

Repeated narcotization by this method at intervals of several days; acclimatization to cold or to CO_2 for several days; or previous thyroid feeding; renders the rat partially or completely resistant to the narcosis induced by 11% CO_2 at 5°C.

A state of depression somewhat similar to, but not identical with, that described above may be produced in rats by exposure to low oxygen tensions (10%)

or to high oxygen tensions (4 atmospheres) at an environmental temperature of 5°C

It is believed that these experiments furnish additional evidence to support the view that a sudden and well marked increase in the tissue tension of CO_2 produces a definite, if temporary, decrease in the total oxidative metabolism and a parallel reduction in activity of certain body tissues including some portions of the nervous system

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THE STIMULATING ACTION OF COLCHICINE ON PITUITARY-INDUCED OVULATION OF THE FROG

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The peculiar property of colchicine of arresting mitosis in the metaphase has formed the basis of its wide application as an aid in the study of the action of administered hormones. The growth effects of oestrogens, androgens, progesterone, pituitary extracts, gonadotropic substances and desoxycorticosterone, have all been determined when given along with colchicine. In these instances the alkaloid has been employed primarily to emphasize the known reactions of the administered hormones, to assist in determining their minimum effective doses and the time of earliest response.

Few workers, however, have studied the possible potentiation of hormone actions by means of colchicine. Havas (1) found that if young bitterling males were placed in colchicine solutions "nuptial" colors developed in approximately the same time (20 hours) as those receiving intraperitoneal injections of testis extract. Combination of the two treatments reduced the time to 1.5 hours. Mann (2) reported an augmentation of the effect of follicular hormone and testis extract on the oxygen consumption by fish by means of the alkaloid. Higbee (3) found that the injection of colchicine into 24-hour chick embryos resulted in comb and wattles approximately twice the normal size—possibly as a result of a potentiation of hormonal effects.

These indications of a synergism between colchicine and endocrine substances led us to examine the effect of the alkaloid and other substances on pituitary-induced ovulation. The ovary of the frog is especially convenient for such a study since ovulation can be induced by the action of pituitary in vitro (4, 5).

Materials and methods. All experiments were carried out between January 15 and March 15 on large female frogs (*Rana pipiens*) collected in New York and Vermont.

For the *in vitro* studies the ovaries were carefully dissected out and each suspended in a total volume of 30 cc. of Ringer's solution containing frog pituitary together with the substance or substances to be tested. Female pituitaries only were used. Usually a large number of glands (24–72) were removed fresh, finely macerated in a small amount of distilled water, and taken up in a large volume of Ringer's. The equivalent of 2 pituitary glands was then pipetted into a Petri dish into which one ovary was placed. By this means fluctuations in potency of individual pituitary glands was eliminated. Each experiment was allowed to run 33 or 36 hours and the number of extruded eggs counted. By this time egg extrusion is practically finished and putrefaction has not ostensibly started. Records were kept of the temperature at the beginning, during, and at the termination of experiments. The greatest temperature range for a single experiment was 17° to 21°C.; and the extremes for the whole experimental period were 17° and 22.5°C.

The pH of the Ringer's solution was not significantly altered by the substances used except in the case of 1% colchicine which was definitely acid. However, immersion of the ovary in this solution brought it to approximate neutrality. No significant difference in the pH of the experimental and control solutions containing the ovaries was encountered in measurements made at the conclusion of the experimental period The pH in most cases was found to be 7 0–7 3, the extremes being 6 7 and 7 4 Measurements were made by means of the glass electrode

Two samples of colchicine were studied (*1*) a fraction obtained from Eimer and Amend (m p 137–141°C (corr)), with which most of the work was done, and (*2*) a highly purified fraction obtained through the kindness of Eli Lilly & Company, (m p 156–157°C (corr)) No special precautions were taken to protect the colchicine solutions from light other than to store them in the ice box between experiments Indication of deterioration was not found when a freshly prepared solution was compared with one of the same concentration, 6 weeks old This is in keeping with Blakeslee and Avery's (6) finding that colchicine is quite stable in solution

DIFFERENCES BETWEEN LEFT AND RIGHT OVARIES Although the difference between the left and right ovary of a pair is somewhat incidental to the main subject of this paper it might well be discussed at this point. It was observed, after a number of experiments had been made, that the left partner of a pair of ovaries was frequently larger than the right In view of this it was possible that with any given experimental treatment the left ovary would extrude more eggs than the right Since the number of eggs extruded was the response employed for testing the action of colchicine and the other substances, it became important to determine the equivalence of right and left ovaries in this regard Each ovary of 21 pairs was treated with the equivalent of 2 female frog pituitaries plus 5 × 10^{-5} concentration of colchicine in 30 cc of Ringer's solution. The number of eggs extruded by left and right ovaries was counted after a 36-hour interval The results are summarized in table 1 In 18 of the 21 cases more eggs were released by the left ovary In the other three the differences were not striking, the total for the three left ovaries being 1232 eggs as compared with 1271 for the right ovaries

TABLE 1

Number of eggs released by, and weights of, left and right ovaries

Values are arithmetic means ± estimated standard error of mean

	NO OF PAIRS	LFT OVARY	RT OVARY	MEAN DIFFERENCE	VALUE OF P AND SIGNIFICANCE
Aver no of eggs extruded	21	581 ± 33	476 ± 29	105 ± 20	10^{-6} S
Aver wt of ovaries (gm)	20	5 72 ± 0 24	4 30 ± 0 17	1 42 ± 0 16	10^{-12} S

The individual weights of left and right ovaries were next compared Twenty pairs were examined and in no case was the right partner as heavy as the left (table 1) Occasionally the discrepancy is very striking, *e g*, in one pair, not included in the table, the left ovary weighed 14 3 grams, the right 4 2 grams The ratio of the total weights for left and right ovaries is 1 3 The ratio of the total eggs released for left and right ovaries is 1 2 The difference in the number of eggs extruded by right and left ovaries is thus apparently to be correlated with a difference in weight (and accordingly the number of ripe ova) between the two members of the pair.

POTENTIATION OF PITUITARY-INDUCED OVULATION IN THE ISOLATED OVARY. The effect of 9 separate concentrations of colchicine on pituitary-induced ovulation has been studied. Data relating to the number of pairs of ovaries examined in each separate concentration, total number of eggs released at the end of 33 hours, and the number of left and right ovaries recorded are given in table 2. The average number of eggs released in the different dilutions is graphically represented in figure 1. The equivalent of 2 female pituitary glands was used throughout and the total volume of fluid in each dish was 30 cc. Ringer's solution without sodium bicarbonate was generally used, but in two series of experiments where it was added the results differed in no observable manner from the others. In our preliminary studies we were unaware of the difference between left and right ovaries and no record was kept as to which member of a pair was placed in the colchicine solution (table 2, columns 2 and 3). In all subsequent work left and right ovaries were, as far as possible, equally divided between experimental and control groups.

TABLE 2

The action of colchicine at various concentrations on pituitary-induced ovulation in vitro. 2 female pituitaries per ovary

COLCHICINE CONCENTRATION	PAIRS OF OVARIES STUDIED	OVARIES IN COLCHICINE LEFT + RIGHT	TOTAL NO. OF EGGS EXTRUDED		RATIO: COLCHICINE-TREATED / CONTROL
			Colchicine-treated	Control	
10^{-8}	6	3 + 3	1,841	2,265	0.8
10^{-7}	9	4 + 5	1,910	1,654	1.2
10^{-6}	9*	3 + 3	4,025	1,345	3.0
5×10^{-6}	9	3 + 3	4,943	2,737	1.8
10^{-5}	9	3 + 3	4,980	1,967	2.5
5×10^{-5}	9	3 + 3	5,251	2,062	2.5
10^{-4}	9	3 + 3	5,133	2,646	1.9
10^{-3}	9	3 + 3	4,082	2,572	1.6
10^{-2}	3	2 + 1	2	650	0.0

* One pair of ovaries in the dilutions 10^{-6}–10^{-3} inclusive, received 1.83 pituitaries.

It is evident from an examination of the recorded data that a 10^{-2} solution of colchicine decidedly inhibits pituitary-induced ovulation; concentrations of 10^{-8} and 10^{-7} appear to have no significant action; the other six dilutions potentiated the pituitary effect. If the total number of eggs released is used as the criterion of effectiveness, then 5×10^{-5} is the optimum concentration of the dilutions studied. The greatest ratio of eggs extruded (colchicine treated/control) was obtained with the 10^{-6} solution, but here the number of control eggs released was the lowest for all groups—with the exception of the 10^{-2} concentration where 3 pairs of ovaries only were studied—and as the ratio is altered greatly by this number (see below), little significance can be attached to it.

Not only was the total number of eggs freed at the end of the 33-hour experimental period recorded, but counts were also made in many cases, (12 pairs) at 3-hour intervals beginning with, or shortly before, the extrusion of the first eggs and continuing until the experiment was concluded. By this means we were able to demonstrate that colchicine-treated ovaries: (*1*) start extruding eggs earlier than the non-treated partners and (*2*) release eggs at a greater rate. A typical result is shown in the form of a graph in fig. 2 and a representative photograph in fig. 3. The picture was taken 24 hours after treatment began and shows clearly the larger number of eggs released from the colchicine-treated ovary which, in this case, is the right ovary and is visibly smaller than its left control.

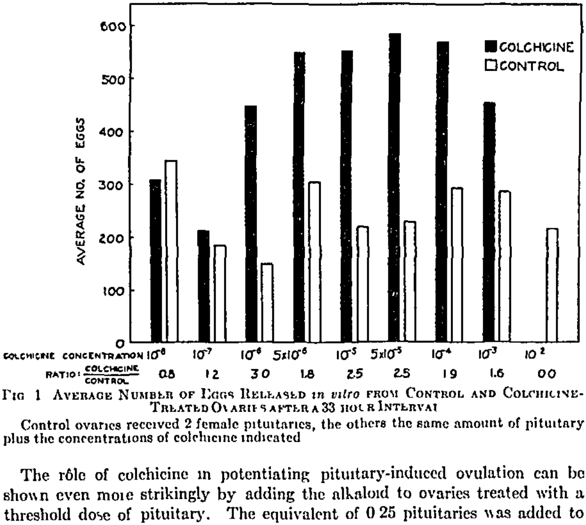

Fig. 1. Average Number of Eggs Released *in vitro* from Control and Colchicine-Treated Ovaries after a 33 hour Interval

Control ovaries received 2 female pituitaries, the others the same amount of pituitary plus the concentrations of colchicine indicated

The rôle of colchicine in potentiating pituitary-induced ovulation can be shown even more strikingly by adding the alkaloid to ovaries treated with a threshold dose of pituitary. The equivalent of 0.25 pituitaries was added to one member of a pair of ovaries in 30 cc. of Ringer's containing colchicine in a concentration of 10^{-4}, while the other member received the same concentration of pituitary but no colchicine. Counts were made after 32 hours. In experiments with 8 pairs of ovaries (table 3) the colchicine-treated ovaries released a total of 1351 eggs as compared with 38 eggs from the ovaries which did not receive colchicine *i.e.*, a ratio of 35.6 (see reference to ratio above). Six of the 8 ovaries which received no colchicine failed to extrude eggs.

The possibility of the induction of ovulation in vitro by colchicine in the ab-

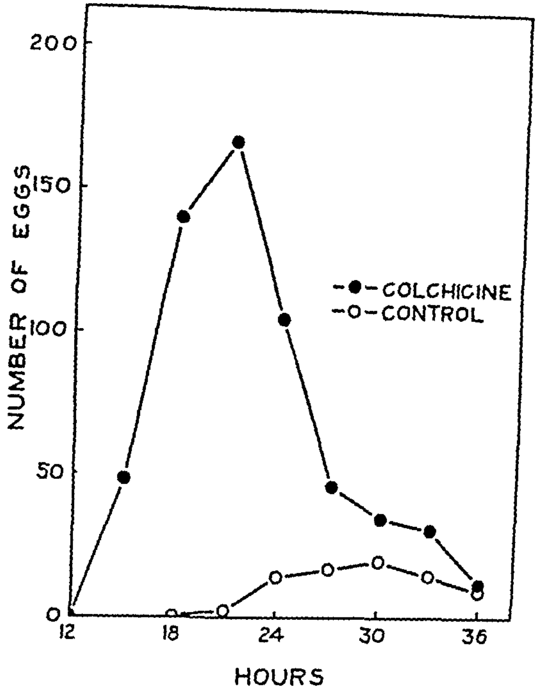

FIG. 2. NUMBER OF EGGS EXTRUDED BY TWO OVARIES OF A PAIR, ONE OF WHICH WAS TREATED WITH PITUITARY PLUS COLCHICINE AND THE OTHER WITH PITUITARY ALONE (CONTROL)

The points indicate the number of eggs released during a 3-hour interval. Colchicine concentration: 5×10^{-4}. 2 female pituitaries in 30 cc. of Ringer's solution on each ovary.

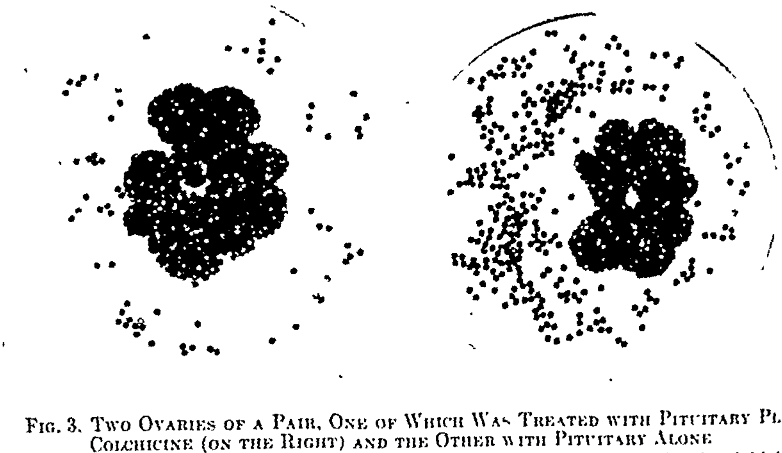

FIG. 3. TWO OVARIES OF A PAIR, ONE OF WHICH WAS TREATED WITH PITUITARY PLUS COLCHICINE (ON THE RIGHT) AND THE OTHER WITH PITUITARY ALONE

Photograph taken 24 hours after beginning of treatment Recrystallized colchicine used in concentration of 2×10^{-5}. The equivalent of 2 female pituitary glands in 30 cc. of Ringer's solution added to each.

sence of pituitary has been studied In experiments in which ovaries were immersed in 10^{-5} and 5×10^{-5} colchicine it was found that of 14 ovaries subjected to colchicine treatment 10 released no eggs and 4 a total of only 20 eggs Colchicine alone, therefore, has little or no direct action on ovulation

POTENTIATION OF PITUITARY INDUCED OVULATION *in vivo* After the demonstration of the augmentative effect of colchicine on pituitary induced ovulation in vitro an attempt was made to obtain similar results in the intact animal

TABLE 3

Colchicine potentiation of a sub optimal dose of pituitary (0.25 pituitaries per ovary) as measured by number of eggs released after 32 hours

EXPERIMENT	EGGS RELEASED PER OVARY	
	Colchicine treated	Control
1	212	6
2	30	0
3	172	0
4	0	0
5	30	0
6	81	0
7	113	0
8	713	32
	1 351	38

TABLE 4

Colchicine stimulation of ovulation in the intact animal

Values are arithmetic means ± estimated standard error of mean

GROUP	NO OF FROGS	DURATION OF EXPERIMENT	AVER WT OF OVARIES AT AUTOPSY		DIFF OF MEANS	VALUE OF P AND SIGNIFICANCE
			Controls	Colchicine		
		hours				
I	3	74	15.06 ± 0.89	7.26 ± 3.13	7.80 ± 3.27	0.1 -0.2 N S
II*	4	47	8.08 ± 0.92	7.18 ± 2.92	0.90 ± 3.06	0.7 -0.8 N S
III	10	60–61	8.36 ± 2.00	2.28 ± 0.78	6.08 ± 2.23	0.02 -0.05 S
I III	17		9.48 ± 1.30	4.31 ± 1.13	5.17 ± 1.72	0.001-0.01 S

* Includes one animal that had died

Seventeen pairs of frogs, matched in weight were studied in three groups The results are summarized in table 4 One animal of each pair received intraperitoneally the equivalent of one half pituitary in 1 cc of Ringer's plus 0.5 cc of 5×10^{-4} colchicine, the other the same amount of pituitary plus 0.5 cc of Ringer's The frogs were killed 74 hours (group I), 47 hours (group II) and 60–61 hours (group III) later and the weight of the ovaries taken as an index of the number of eggs released The ovaries were carefully dissected free, the excess fluid removed on blotting paper, and weighed to the nearest 0.01 gram

Although the results obtained in the first two groups are suggestive of colchicine potentiation, they are *not statistically* significant. The data of Group III, however, are significant and also the results from all groups when treated together. P values are included in the table.

A photograph of the ovaries at autopsy of a pair of animals from Group III is shown in fig. 4.

The dose of colchicine may have been unnecessarily high: one animal died in each of the first two groups and the viscera of the colchicine-injected frogs were in some cases more congested than that of the controls.

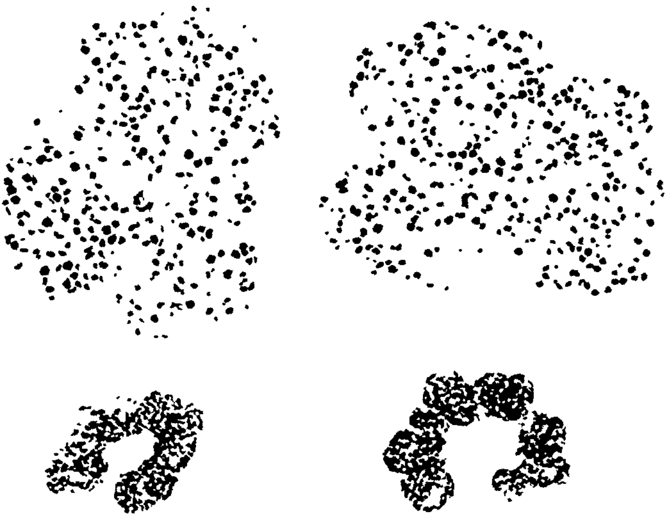

Fig. 4. The 2 lower ovaries were taken from a frog 60–61 hours after an injection of 0.25 female pituitaries plus 0.5 cc. of 5 × 10^{-4} colchicine. The upper ovaries were taken from a frog of approximately the same weight which had received the same amount of pituitary but no colchicine. *A portion of the upper right ovary which has released eggs appears* grayish in the photo. Nearly every ripe egg has been released by the lower pair of ovaries.

It may be mentioned that to demonstrate colchicine potentiation by this method a sub-optimal dose of pituitary should be used, since an optimal dose would release all, or nearly all, of the ripe eggs from the ovary even in the absence of colchicine and thus no difference would be apparent. This occurred in 2 cases in Group III, table 4.

An attempt was made to stimulate the pituitary *of intact animals by means* of colchicine. Eight animals, 53–65 gm. in weight were used—four were given 0.5 cc. of 5 × 10^{-4} colchicine intraperitoneally and four, of approximately similar size, 0.5 cc. of Ringer's. One animal was found dead after 72 hours. Its control was killed and both autopsied. No free eggs were found in either animal. Similarly, no indication of ovulation was obtained in the other 3 colchicine-injected animals when autopsied two hours later This phenomenon has not been studied further

FERTILIZATION In view of the inhibitory effect of colchicine on cell division it was of interest to determine whether eggs released from the ovary of the intact animal as a result of the combined action of pituitary and colchicine could be fertilized and would undergo division Three frogs each received an injection of 3 female pituitaries and 0 2 cc of 5×10^{-4} colchicine Three control animals received only the pituitaries A sperm suspension was prepared by macerating the testes of untreated frogs in diluted Ringer's solution Eggs released from the ovaries of both colchicine treated and control frogs were stripped into the sperm suspension Nearly 100% of the eggs from the colchicine treated females were fertilizable as indicated by their orientation, but none was observed to divide Fertilization and division were normal in eggs from the controls Serial sections of the eggs from the colchicine treated animals have not yet been studied, however, inhibition of normal maturation is to be expected

TABLE 5

The effect of certain chemical agents on pituitary induced ovulation in vitro Egg counts made after 33 hours

AGENT USED	CONC N	NO OF EXPTS	TOTAL NO OF EGGS RELEASED		RATIO EXPTL / CONTROL
			Exptl	Control	
Papaverine	10^{-5}	9	1 641	2,442	0 67
Adrenaline	$0\ 46 \times 10^{-5}$	6	866	2,027	0 44
Adrenaline	$0\ 92 \times 10^{-5}$	6	542	1,844	0 29
*Acetyl choline	10^{-5}	5	1,110	1,185	0 93
Eserine	2×10^{-5}				
*Acetanilid	$0\ 34 \times 10^{-5}$	6	1 291	1,303	0 99

* Total volume 40 cc

THE ACTION OF OTHER CHEMICAL AGENTS ON PITUITARY INDUCED OVULATION Studies of the action of papaverine, adrenaline, acetyl choline and eserine, and acetanilid on pituitary induced ovulation were made Table 5 summarizes the results of these experiments As in previous experiments 2 female pituitaries per ovary were used as the inciting dose and egg counts were made after 33 hours

No derivatives of colchicine were available for investigation and there appeared to be no common organic compounds closely resembling colchicine in structure Papaverine was considered because of its being a fairly complex alkaloid and possessing four methoxy groups like colchicine It was found to inhibit the action of pituitary (table 5)

Raymond Hamet (7) and Busquet (8) found that colchicine augmented the motor and inhibitory effects of adrenaline and in view of these reports adrenaline was studied Unlike colchicine, however, it definitely inhibited the release of eggs in the isolated ovary (table 5) In the first experiment (6 cases) no precautions were taken to protect the solutions from light In a repetition of this experiment, however, the dishes were kept in the dark and adrenaline was added at the beginning of the experiment and then again 12 hours later when ovulation was just beginning. In this second experiment the ovaries were immersed in 30 cc. of Ringer's and 1.5 cc. of a 0.92×10^{-4} solution added and then 12 hours later another 1.5 cc., the final volume being 33 cc. Both solutions were inhibitory, the second slightly more than the first; but as more adrenaline was used in the latter this difference is not surprising.

As we had somewhat unexpectedly obtained inhibition with adrenaline it was felt that it would be interesting to investigate the action of acetyl choline. There was no appreciable action (table 5). Acetanilid was used because it, like colchicine, possesses an acetyl-N grouping. It was found neither to stimulate nor to inhibit pituitary-induced ovulation (table 5).

Discussion. It has been shown in the present study that colchicine will potentiate the action of anterior pituitary substance. That this effect is a true potentiation and not a simple algebraic summation of the individual actions of anterior pituitary and colchicine is obvious from the demonstration of the infinitesimal action of colchicine when used alone. The mechanism of this positive synergism was not studied, but two possible explanations present themselves: (*1*) that colchicine renders the ovary more susceptible to pituitary, (*2*) that it acts directly on the macerated pituitary tissue causing it to liberate more of the active principle or possibly modifies the latter so that it becomes more potent. If the action is primarily on the ovary, injection of colchicine alone into the normal intact animal would be expected to produce ovulation only in the presence of sufficient pituitary substance in the blood, since it has been shown that colchicine alone is ineffective on the isolated ovary in the absence of pituitary. Injecting frogs with colchicine did not result in the release of eggs. Perhaps, however, as the mating season is approached enough pituitary hormone would be present in the blood so that a colchicine effect would be demonstrable. If colchicine has a direct action on the intact pituitary, it clearly does not release sufficient hormone to cause ovulation.

Six concentrations of the alkaloid were found to augment the action of pituitary, namely 10^{-3}, 10^{-4}, 5×10^{-5}, 10^{-5}, 5×10^{-6} and 10^{-6}, and a rough relationship was found between these concentrations and the number of eggs extruded. The effect increased from the strongest active solution to reach a peak at 5×10^{-5} and then decreased to the 10^{-7} concentration, which was inactive. By considering other factors, e.g., aeration, temperature, the amount of pituitary, etc., this relationship might be made more decisive and the method adaptable to the biological assay of colchicine. Moreover, Ryan and Grant's (5) suggested method for the assay of pituitary might be made more sensitive by the use of colchicine.

Papaverine (10^{-5}) and adrenaline (0.46×10^{-5} and 0.92×10^{-5}) caused inhibition of ovulation in the isolated ovary but the possibility of potentiation in different strengths of solution must be borne in mind. However, it is to be noted that these agents proved inhibitory in concentrations of an order that were strongly augmentatory in the case of colchicine.

SUMMARY

1. Colchicine in concentrations of 10^{-3}, 10^{-4}, 5×10^{-5}, 10^{-5}, 5×10^{-6} and 10^{-6} potentiated the effect of pituitary in causing ovulation in the isolated frog's ovary. 10^{-2} colchicine inhibited ovulation. 10^{-7} and 10^{-8} solutions were without effect.

2. Papaverine and adrenaline inhibited pituitary ovulation in the excised ovary. Acetyl choline with eserine, and acetanilid failed to modify the pituitary effect.

3. Ovulation by injected pituitary in the intact animal was augmented by the simultaneous administration of colchicine.

4. Colchicine alone failed to incite egg release both *in vitro* and *in vivo*.

5. The left ovary was found to be larger than the right ovary and to extrude more eggs. There was a close correlation between ratio of weights for left and right ovaries and the ratio of number of eggs extruded by the left and right ovaries.

6. Eggs from colchicine-injected frogs failed to divide when fertilized with normal sperm.

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STUDIES ON ANTIMALARIAL DRUGS

THE DISTRIBUTION OF QUININE IN THE TISSUES OF THE FOWL

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The distribution of quinine has been studied in the tissues of the cat (1), dog (1, 2, 3, 4), rabbit (5, 6, 7) and guinea pig (2, 6, 7). However, these species are not susceptible to infection by malaria parasites, hence they are unsuitable for studies intended to determine whether the tissue metabolism of quinine is affected by the presence of the infection. Furthermore, interpretation of the action of quinine in malaria must be based on knowledge of the metabolism of the drug in those species susceptible to malarial infections. Despite the extensive use of avian malaria in chemotherapeutic studies, the metabolism of quinine in either normal or infected birds has not been reported. The present paper deals with the distribution of quinine in the tissues of normal chickens after both oral and intravenous administration. The effect of experimental avian malaria will be reported in a later communication.

Materials and methods. White Leghorn roosters were used in all experiments. The calculations of the quinine administered are based on the amount of free alkaloid. When oral administration was used, quinine dihydrochloride was given in gelatine capsules. In the intraveneous studies, a 1% solution of quinine (as the hydrochloride) in distilled water was injected slowly into the wing vein. Quinine in the blood and tissues was determined by the micro-method recently described by Kelsey and Geiling (8).

For blood studies, blood was collected from the jugular vein using potassium oxalate as an anti-coagulant. The oxalated blood was centrifuged and the plasma siphoned off. The upper portion of the cells (mostly white) was separated from the bulk of the red cells, resuspended in plasma and centrifuged in a 15 cc. graduated centrifuge tube. The plasma was drawn off again by suction; the relative volumes of the white and red cells noted, and the mixture analysed for quinine. Since the concentration of quinine in the red cells was determined in a separate sample, the concentration in the white cells could be calculated.

Results. *Distribution of quinine after oral administration.* Our studies on the distribution of quinine after oral administration can be divided into three parts: first, the concentration in the blood after a single dose; second, the distribution in the tissues after repeated doses; and third, the distribution in the tissues after a single dose.

The purpose of the first experiments was to determine the extent of individual variation in the rate of absorption and detoxification of quinine. Six chickens weighing approximately 2.5 kg. were given 400 mgm./kg. by mouth. Blood samples were obtained either from the wing vein or by heart puncture at intervals of from 15 minutes to 21 hours after administration. The results are presented in figure 1 in which the blood concentrations for each bird are given in mgm./liter. The extreme variation in blood levels after oral administration of quinine makes it impossible to predict the time at which the maximum blood level will be reached Not only did the time at which the peak occurred vary from 3 to more than 8 hours, but the maximum blood level varied from 5 to 25 mgm /liter

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[1] John J. Abel fellow in Pharmacology.

The purpose of the second group of experiments was to determine the extent of accumulation of quinine in the tissues after repeated doses Six young roosters were given quinine orally according to the following schedule —100 mgm /kg for the first four days 150 mgm /kg for the sixth seventh and eighth days 300 mgm /kg for the tenth eleventh twelfth, fourteenth, fifteenth and sixteenth days No quinine was given on the fifth, ninth and thirteenth days The birds were sacrificed at intervals of 3, 6, 12 24, 36 and 48 hours after the last dose

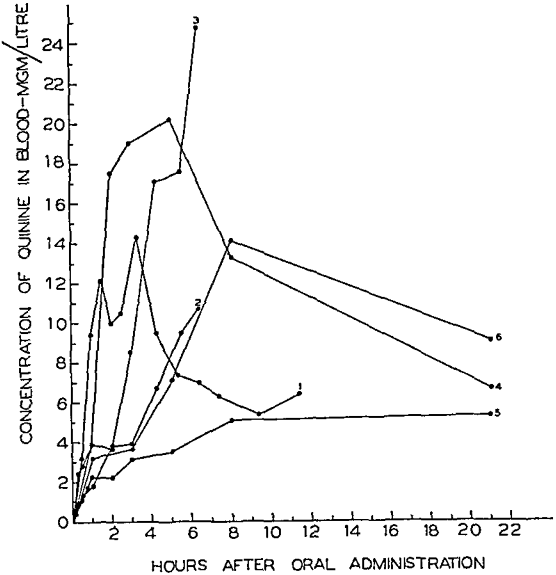

Fig 1 The Concentration of Quinine in the Blood of Six Fowls after Oral Administration of 400 Mgm/Kg to Each

Birds no 2 and 3 died during the course of the experiment

In the third group of experiments individual variations in the tissue distribu tion of quinine after a single oral dose were studied. Four birds were given 300 mgm./kg. of quinine and sacrificed 12 hours later. The data obtained from the last two groups of experiments are presented in figure 2. The circles represent the values obtained after repeated oral administration, while the bars represent the values obtained after a single oral dose. The high degree of individual variation after oral administration is as apparent in the tissue studies as in the blood studies (fig. 1).

Regarding a possible accumulation of quinine, it can be seen that even after a prolonged administration, by far the greater portion of the drug is detoxified each day, since very little was found in the tissues of the chickens sacrificed 24 hours or more after the last dose.

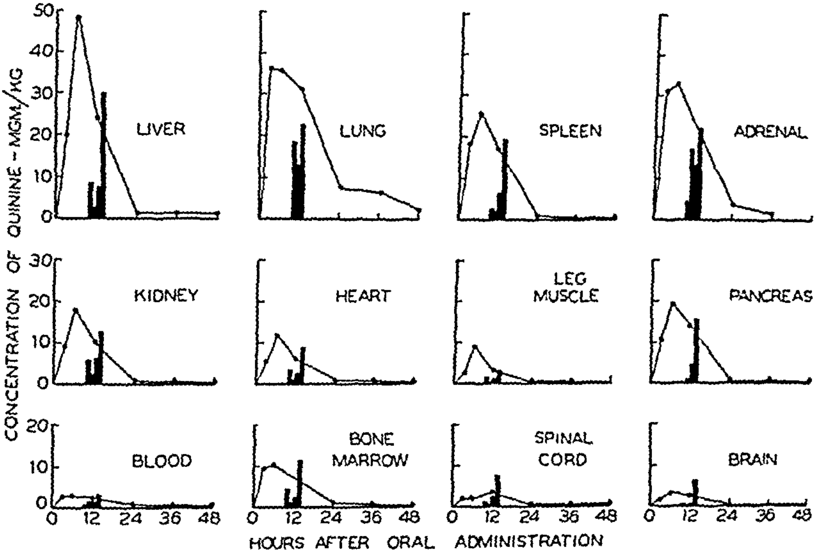

FIG. 2. CONCENTRATION OF QUININE IN THE TISSUES OF THE FOWL AFTER ORAL ADMINISTRATION

Each circle represents one bird killed at the indicated time after the last of a series of doses (see text). Each bar represents one bird killed 12 hours after a single dose of 300 mgm/kg.

Distribution of quinine after intravenous injection. The extreme individual variations in absorption and distribution of quinine in the tissues of the chicken after oral administration prompted a study of the distribution after intravenous injection. Seven birds were given 10 mgm./kg. of quinine by injection into the wing vein and were sacrificed at intervals of 10, 15, 60 (3 birds), 120 and 240 minutes. The results of tissue analyses are set forth in figure 3. The solid bars represent three chickens killed one hour after injection. Not only do these chickens show approximately the same concentration of quinine in a given tissue but the amounts present fall within the range expected from chickens killed at earlier and later periods (represented by the solid circles). Since the variations from bird to bird in the intravenous series is much less than in the oral series (fig 2), the latter is probably due in large part to individual differences in the rate of absorption from the gut

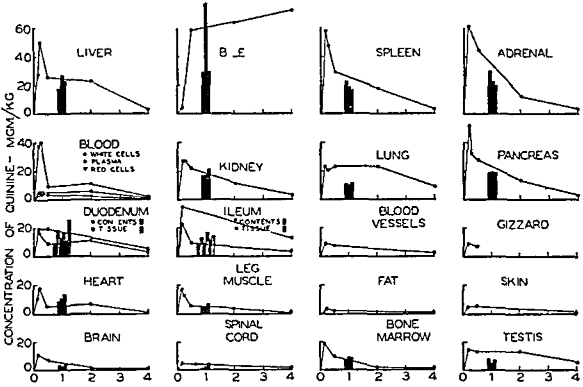

Fig 3 Concentration of Quinine in the Tissues of the Fowl after Intravenous Administration of 10 Mgm/Kg

Each circle represents one bird killed at the indicated time after the injection Each bar represents one bird killed one hour after the injection

TABLE 1

Concentration of quinine in the tissues of the chicken, dog and rabbit one hour after the intravenous injection of 10 mgm /kg of quinine

TISSUE	CHICKEN (AV OF 5)	DOG	RABBIT (AV OF 2)
	mgm of quinine per kg of tissue		
Blood	3	3	0 3
Liver	22	23	1
Bile	29	10	5
Lung	13	37	54
Kidney	17	14	8
Spleen	21	29	10
Heart	10	8	2
Muscle	5	4	2
Brain	3	3	1

For a short interval after intravenous injection, the concentration of quinine in the red blood cells was slightly higher than in the plasma However, in none of five birds was the concentration in the red blood cells higher than in the plasma one hour after the injection. The white blood cells after showing a remarkably high initial concentration of quinine, maintained for some hours a concentration considerably higher than the red blood cells or plasma. Unpublished experiments on the human being after oral quinine also show appreciably higher concentrations in the white blood cells.

The rapid removal of quinine from the blood is reflected by the high concentrations in the liver, kidney, lungs, spleen, pancreas and adrenals ten minutes after the intravenous injection, while the blood in this interval had dropped to a more or less constant level. The increasingly large amounts found in the bile indicate that the liver plays an important rôle in the elimination of the alkaloid. However, since appreciable amounts are present in the contents of the small intestine during the first ten minutes, when the content in the bile is low, it is apparent that in the chicken as in other species, quinine is excreted directly into the bowel.

In table 1, comparative values are given for tissue concentrations in the chicken, dog and rabbit, one hour after the intravenous administration of 10 mgm./kg. The concentrations observed are more or less in inverse ratio to the ability of the tissues of that species to destroy quinine *in vitro*. Details of these experiments will be reported later.

SUMMARY

The distribution of quinine in the blood and tissues of the fowl has been determined after single and repeated oral doses and after intravenous injection.

The blood levels after oral quinine show great individual variation both as regards the peak concentration and the time at which it occurs. Individual variations are also reflected in the tissue concentrations after oral administration.

Quinine is not accumulated in the tissues of the fowl even after long continued administration. Practically all of the quinine has disappeared from the tissues twenty-four hours after the last dose.

Immediately after the intravenous injection of quinine the concentration in the red blood cells is greater than in the plasma. Within an hour, the ratio is reversed. The white cells always show appreciably higher amounts of quinine than either the red cells or the plasma.

Quinine is especially concentrated in the liver, spleen, adrenal, pancreas and white blood cells, to a lesser extent in the bone marrow, lung, heart, testis, and kidney, while in the brain, spinal cord, skin, depot fat and blood contain relatively little. The distribution is essentially the same after either oral or intravenous administration. The greater individual variation in birds given oral quinine is probably due to differences in rate of absorption from the gut.

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BLOOD PRESSURE AND RESPIRATORY CHANGES PRODUCED BY STRYCHNINE CONVULSIONS

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The various blood pressure effects of subconvulsant and convulsant dosages of strychnine have been analyzed (1, 2). However, no cogent pharmacodynamic explanation has been advanced for the various changes in blood pressure which occur during and following strychnine convulsions. The physiologic mechanisms responsible for such changes during metrazol convulsions have been analyzed and were shown to differ in man and the dog (3). Employing strychnine as the convulsant, a comparable investigation was undertaken using dogs and cats as experimental animals. For obvious reasons the studies could not be extended to man as was done with metrazol.

The arterial pressure changes associated with convulsions are the resultant of the simultaneous activation of a number of systems, viz., the sympathetic nervous system, the parasympathetic nervous system and the skeletal muscular system, including the changes in intra-thoracic and intra-abdominal pressures resulting from skeletal muscular activity.

Methods. After strychnine dosages varying from 0.1–0.2 mg./kg., intravenously, convulsions were induced in 26 dogs and 7 cats by slapping the hind quarters or the head region 5 times. Approximately the same degree of force was applied each time. This method of stimulation was considered suitable since arterial pressure changes which occurred as the result of spontaneous convulsions were similar to those produced by convulsions which were elicited by slapping.

In unanesthetized animals surgical procedures were performed under local anesthesia with procaine. Other solutions were injected into the exposed femoral vein and an adequate airway was maintained throughout certain experiments either by previous cannulation or intubation of the trachea.

The arterial pressure was continuously recorded from either the femoral or carotid artery by means of the hypodermic manometer (4, 5). In order to estimate the contribution of the intra-thoracic and intra-abdominal pressures to the arterial pressure, differential manometric measurements were made. One balloon was placed in the abdomen and another in the thorax with an air tight closure made around the tube leading from each to the front of differential manometers (6). The balloons were filled with water through a T tube and sufficient additional fluid was introduced so as to fill the rubber tubing to within 3 cm. of the front of the differential manometer. Respiratory rate and amplitude were recorded by a rubber membrane optical manometer connected to a T tube in the trachea. Total blood oxygen and carbon dioxide were determined by the Van Slyke and Stadie Method (7) in blood collected from the femoral artery.

The relative contribution of the sympathetic and parasympathetic nervous systems and the skeletal muscular system to the resultant blood pressure changes occurring during and after convulsions were estimated by the judicious employment of suitable drugs and operative techniques. These included atropine sulfate 0.1–0.2 mg./kg., ergotoxine ethanesulfonate 2 mg./kg., beta-erythroidin hydrochloride 2-5 mg./kg., bilateral vagotomy in the neck and high spinal anesthesia (C_3-C_4) with procaine hydrochloride.

Figures employed are based upon at least 3 and in some cases of 5 experiments.

RESULTS AND DISCUSSION. The injection of convulsant doses of strychnine did not result in changes in the blood pressure until either the nervous or the skeletal muscular component of a convulsion was present. This was equally true in animals narcotized with ether or alcohol, where it was necessary to use 3-6 times the M.L.D. in order to produce convulsions.

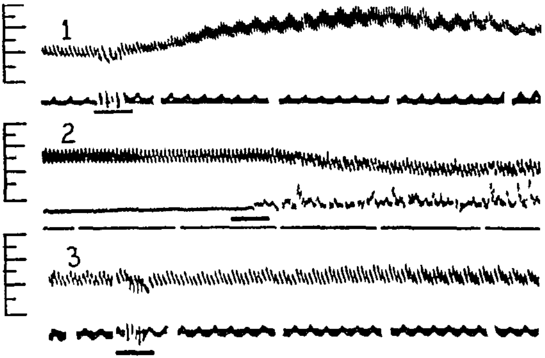
During strychnine convulsions, the blood pressure changes vary in different animals of a given species. *This fact deserves primary consideration in evaluating* the contribution of a particular mechanism to the composite effect appearing in the intact animal. Isolation of the physiologic factors responsible for a particular effect has been attempted.

It is necessary to recognize that two chief systems contribute to the blood pressure changes observed during a convulsion, viz., the nervous and skeletal muscular systems. The fact that the response of the skeletal muscular system was largely or completely abolished in some of these experiments by beta-erythroidin (fig. 1) or by high spinal anesthesia (fig. 4) does not necessitate the conclusion that the nervous component of the convulsion was absent or reduced in amount. During these types of experiments, there were sympathetic and parasympathetic responses even when there was no skeletal muscular activity. This constitutes evidence of a nervous system component of convulsions.

Contribution of the sympathetic nervous system. Cutaneous stimulation of vagotomized, strychninized animals, following beta-erythroidin, caused a gradual and prolonged rise in arterial pressure (fig. 1). This occurred even when the skeletal muscular contribution to the convulsion was practically abolished by beta-erythroidin. Since sympatholytic dosage of ergotoxin abolished this rise (fig. 2) there is no doubt that the elevation shown in figure 1 was due to sympathetic nervous system hyperactivity.

Since ergotoxin is known selectively to abolish augmentory sympathetic effects, it is a reasonable conjecture that the decrease in arterial pressure shown in figure 2 was due to the continued liberation of Sympathin I. However, this explanation for this decrease in pressure is untenable, since paralysis of skeletal musculature by beta-erythroidin abolished the fall (fig. 3). Also, it is not necessary to postulate that beta-erythroidin antagonizes Sympathin I, since it was still possible to produce an epinephrine reversal in animals which had received both ergotoxin and beta-erythroidin (8). This gradual decrease in arterial pressure (fig. 2) was undoubtedly associated with excessive skeletal muscular activity occurring during clonic and following tetanic convulsions (discussed below). Pharmacodynamically, strychnine and metrazol differ in that after strychnine activation of reflex mechanisms by sensory stimulation is necessary for the appearance of autonomic effects on the cardiovascular system, while after metrazol effects are observable only at the time the drug is injected. In the case of metrazol, the initial convulsion resulting from the drug injection is associated with autonomic activity but subsequent convulsions are not (3).

These results with strychnine confirm the findings of Harmon and McFall (9) and Watanabe (10) with respect to adrenin secretion. Stewart and Rogoff (11) have shown that single large doses of strychnine cause an excessive liberation of epinephrine from the adrenals. Their individual protocols strongly suggest that convulsions are necessary for a marked epinephrine liberation to occur. In addition, Stewart and Rogoff's animals were anesthetized with ether and morphine. This pharmacologic background has been shown by Emerson (12) and Harmon and McFall (13) to cause epinephrine release in itself. The chronic



FIGS 1-3 SIMULTANEOUS CAROTID PRESSURE PULSES AND INTRA ABDOMINAL PRESSURES OF UNANESTHETIZED DOGS

FIG 1 Strychninized vagotomized dog which had received beta erythroidin 10 mg /kg administered over a period of 4 minutes. Cutaneous stimulation by slapping at signal 4 minutes after completion of beta erythroidin injection

FIG 2 Strychninized vagotomized dog which had received ergotoxin 2 mg /kg. Cutaneous stimulation at signal

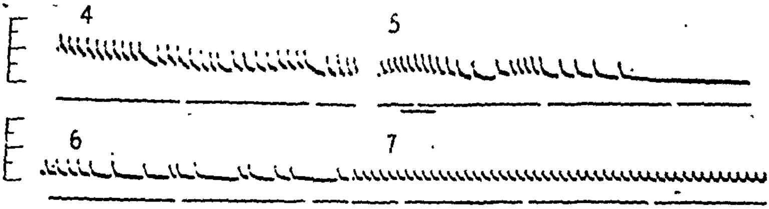
FIG 3 Strychninized vagotomized dog which had received beta erythroidin and ergotoxin. Control record of blood pressure then cutaneous stimulation at signal

In these and all subsequent records, except where indicated differently, the pressure scales are shown in units of 50 mm Hg and the base line is interrupted at 10 second intervals

toxicity studies of Fenton *et al* (14) with strychnine in cats indicate that excessive sympathetic activity may be correlated with pathologic changes. In their animals convulsions could be considered as a possible factor in the production of such marked pathologic disturbances. Undoubtedly strychnine also increases sympathetic activity in non convulsant dosage but to a minor degree, since it is observable only under special experimental conditions (Harned and Cole 15)

Contribution of the parasympathetic nervous system In an adequately aerated strychninized dog which had the sympathetic nervous and skeletal muscular systems inactivated by spinal anesthesia to the level of the third cervical vertebra spontaneous convulsions of head muscles were associated with cardiac slowing and a gradual decrease of arterial pressure (fig. 4). Analysis of the diastolic slope of pulse contours at the beginning and end of the selected record indicates that vasodilation partly caused the decrease in pressure. After convulsions these pulse contours show during diastole an increase in the rate of descent of pressure at any given pressure. This is evidence of peripheral vasodilation in the absence of a mechanism which would tend to restrict the arterial reservoir.

Repeating the cutaneous stimulation of the head resulted in cardiac arrest lasting 15 seconds (fig. 5), but no further extra-cardiac decrease in arterial pressure. During a series of spontaneous convulsions, there was vagal slowing of the heart during each (fig. 6). After atropine administration, similar convulsions of head muscles were associated with no change in cardiac rate (fig. 7). Doubtless, the transient cardiac arrest was vagal in origin.



FIGS. 4–7. FEMORAL PRESSURE PULSES OF STRYCHNINIZED DOG UNDER INFLUENCE OF SPINAL ANESTHESIA TO LEVEL C_5–C_4

FIG. 4. During spontaneous convulsion of head muscles.

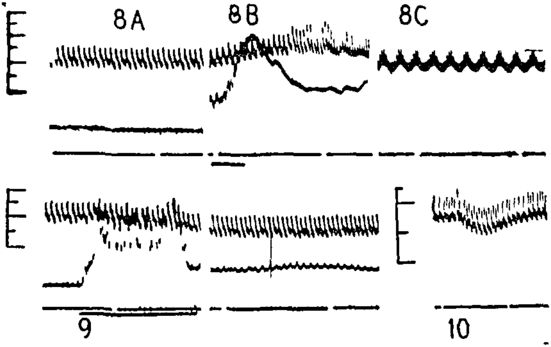
FIG. 5. Before and during convulsions of head muscles after cutaneous stimulation of head indicated at signal.

[illegible] sions, 1½ minutes after effects of convulsion due to [illegible] At break between figures 6 and 7, atropine sulfate [illegible] administration, during convulsions of head muscles after cutaneous stimulation, indicated by signal.

Contribution of the skeletal muscular system. After sympatholytic ergotoxin dosage, cutaneous stimulation of strychninized dogs resulted in convulsions which were accompanied by an *initial passive rise* in arterial pressure followed by a *secondary fall.*

The *initial passive rise* could result from an increase in: *1)* intra-thoracic pressure (3, 5, 16, 17, 18, 19), *2)* intra-abdominal pressure, *3)* peripheral resistance by compression of arterioles and capillaries, and *4)* venous return due to the forcing of blood out of skeletal muscles and the abdominal area. The intra-thoracic pressure frequently contributed as little as 2–6 mm. Hg to this increase in arterial pressure. Since there was no significant rise in intra-thoracic pressure, it is conceivable that either the glottis was open or the expiratory and inspiratory muscles were balanced. The intra-abdominal pressure increased 38–66 mm. Hg and thereby contributed significantly to the *initial passive rise.* This portion of the rise was possibly mediated by: *1)* a compression of the large arteries of the abdomen and *2)* a compression of arterioles and capillaries in the viscera which also may increase peripheral resistance. In addition, peripheral resistance was further increased by compression of vascular beds in the tetanically contracted skeletal muscles Venous return was augmented by the increased intra-abdominal pressure and the tetanic and clonic contractions of skeletal muscles (3, 20) (fig 8) These forces squeezed blood out of peripheral beds into the vessels of the thoracic cage where the pressure was low Doubtless the immediate cardiac acceleration resulted from the increased venous return and the consequent dilation of the right heart and large veins of the thorax (21)

The *secondary fall* followed quickly and was short or long in duration It was associated with tetanic or clonic convulsions and continued after convulsions



FIGS 8–10 PRESSURE RECORDS FROM DOGS

FIG 8 From above downward pressure pulses from femoral artery and ligated femoral vein 8A = control record before a convulsion 8B = effect of convulsion after ergotoxin and strychnine 8C = recovery period after convulsion showing arterial pressure remaining low

FIG 9 From above downward pressure pulses from femoral artery and ligated femoral vein Effects of struggling on arterial and venous pressures Struggling during signal

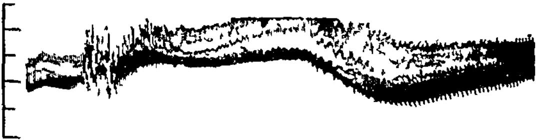
FIG 10 Femoral arterial pressure pulses during momentary convulsions consisting of widespread skeletal muscular activity lasting less than 1 second Arterial pressure scale in units of 50 mm Hg except top division which represents 25 mm Hg

ceased Skeletal muscular activity is necessary since beta erythroidin administration prevented the appearance of the fall (fig 3) This *secondary fall* may have been caused by the following mechanisms which accompany hyperactivity of skeletal muscles *1*) intra muscular hypoxia *2*) excessive vasodilator metabolite production and in addition *1*) excessive antidromic stimulation and *2*) excessive stimulation of vasodilator nerves A similar fall (fig 9) often accompanies struggling in normal or ergotoxinized animals This depressor response is accentuated by strychnine, since both tetanic (fig 2) and momentary (fig 10) convulsions produced a more marked reduction in arterial pressure than resulted from struggling

COMPOSITE EFFECTS OF STRYCHNINE CONVULSIONS IN UNANESTHETIZED ANIMALS. a) *Adequate Aeration.* The blood pressure changes associated with strychnine convulsions in adequately aerated dogs are illustrated in figure 11. These changes can be divided into several phases. The first phase begins with the onset of convulsions and consists of the *initial passive rise.* During clonic convulsions, the blood pressure shows rapid passive fluctuations. As the convulsion becomes tetanic, the blood pressure elevation is maintained. The second phase follows as other mechanisms are activated. Cardiac acceleration occurs and may result from either an increased venous return or sympathetic nervous system stimulation. The arterial pressure rises further and is accompanied by an increase in pulse pressure. These changes result from sympathetic vasoconstriction (fig. 1) and extra-vascular compression, both of which reduce the volume of the systemic arterial reservoir and increase peripheral resistance. The elevated extra-vascular pressure forces a large volume of blood from the peripheral beds and the abdominal reservoirs. The skin reservoirs and the pulmonary bed become engorged and provide emergency reservoirs for this large volume of blood. During this period, pulmonary arterial and venous pressures sometimes increased 45 mm. Hg (8). Since pulmonary engorgement occurred, it is evident that the left heart was unable to transfer all of this increased load of blood into the reduced systemic vascular tree. Skeletal muscular hyperactivity results in vasodilation (fig. 2) which opposes these effects. However, this depressor action is effectively masked by both the excessive sympathetic vasoconstriction and the increased extra-vascular pressure. Tonic convulsions masked the effects of vasodilation more completely than did clonic convulsions, since the latter allow intermittent periods of normal extravascular pressure. Often in the second phase there were intermittent periods of cardiac slowing which may have resulted from activation of depressor reflexes, but were more likely due to central hyperactivity of the parasympathetic nervous system (fig. 5) or peripheral sensitization of cholinergic endings.

The third phase starts with skeletal muscular relaxation and with the concomitant return of extra-vascular pressures to preconvulsive levels. Arterial pressure during this phase may be lower (fig. 8C) or higher (fig. 11) than the preconvulsive level. This is determined by the relative activity of the sympathetic and parasympathetic nervous systems and by the degree of vasodilation secondary to convulsive muscular activity.

The blood pressure changes associated with strychnine convulsions resemble those produced by straining in man (Valsalva's experiment (5)). The *initial passive rise* during convulsions and the first and second phases of straining is chiefly due to extra-vascular reinforcement. The mechanism of the reinforcement differs in the two conditions in that during convulsions there are: *1)* an increase in the intra-abdominal pressure with no significant increase in intra-thoracic pressure, *2)* a decrease in the volume of the systemic vascular tree and extra-thoracic reservoirs, *3)* an increase in venous return and *4)* an active elevation of net pulmonary arterial and venous pressures producing pulmonary engorgement. During straining both the intra-thoracic and intra-abdominal pressures increase so that blood is dammed into the skin and extremities resulting in a decreased venous return and a depletion of the pulmonary reservoir Consequently during convulsions the intense contraction of skeletal muscles of both the abdomen and the extremities contribute largely to the *initial passive rise* while during straining only the increase in activity of abdominal and thoracic muscles contribute significantly to this rise It is evident that the heart is subjected to serious stresses during convulsions, since peripheral resistance is increased and since the intra thoracic pressure does not rise sufficiently to prevent its being overloaded During straining blood is prevented from entering the thorax and no serious stress is placed on the heart It is only immediately after



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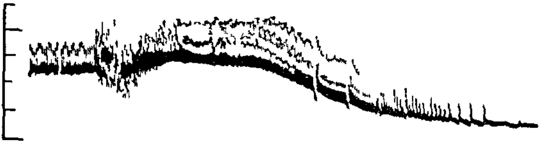

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Figs 11 and 12 Femoral Arterial Pressure Pulses of Unanesthetized Strychninized Dog

Fig cutaneous stimulation

Fig luced by cutaneous stimul e l ne

the third phase of straining is over that any significant overloading of the heart occurs

b) *Inadequate Aeration* Animals which are not adequately aerated generally die after a few convulsions Death appears to be due to cardiovascular collapse, vagal arrest and/or respiratory failure

The blood pressure changes which occur during the first and second phases of the convulsions are essentially similar to those observed in adequately aerated animals (Compare figs 11 and 12) However some striking differences appear early in the third phase In some animals inadequate aeration accentuated the decrease in blood pressure of the third phase so that there was a marked fall below the pre convulsive level and the animal died of cardiovascular collapse (fig. 12). This may be largely due to a greater peripheral vasodilation resulting from skeletal muscular activity and asphyxia, together with cardiac failure.

In other animals there was pronounced vagal slowing of the heart in addition to accentuation of the blood pressure fall. The pronounced slowing of the heart, which often is fatal, substantiates other findings (22) which indicate that asphyxia is conducive to a decrease in acetylcholine destruction and a subsequent hyperactivity of cardiac cholinergic endings. Also, vagal escape could not occur, since blood was pooled in excessively dilated peripheral beds and venous return was not adequate.

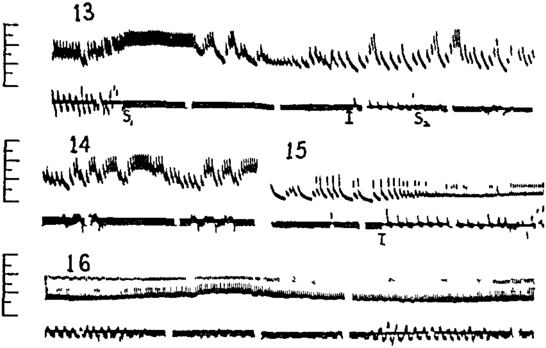
In other animals which were inadequately aerated during convulsions, respiratory arrest occurred during the third phase. The following possibilities may be considered as causes for this arrest: anoxia, acapnia, hyperacidemia, and fatigue of the respiratory center by bombardment with peripheral impulses which are normally subthreshold.

The degree of anoxemia occurring in animals subjected to convulsions was determined in 1 cat and 2 dogs. These animals were unanesthetized and received strychnine sulfate 0.2 mg./kg. intravenously. Dog A had only 1 tetanic seizure which was fatal; dog B and the cat each had 1 tetanic seizure prior to the fatal convulsion. The fatal convulsion lasted 5–8 minutes. At the end of this time the blood pressure had decreased to approximately 18 mm. Hg and the arterial blood oxygen tensions (mm. Hg) were: 18, 10 and 14; total oxygen values (volumes %) were: 7.6, 3.9 and 6.4; and hemoglobin saturation values (%) were: 35, 18 and 30. Total carbon dioxide values (volumes %) of these same blood samples were: 26.7, 32 and 16.4.

The arterial blood oxygen values in these strychninized animals are not as low as those reported by Koehler *et al.* (23) for pigs whose respiratory center failed after 25–29 minutes when they were breathing 5% oxygen in nitrogen. Undoubtedly, respiratory center depression is present with the low arterial blood oxygen values found in the strychninized animals, but this degree of anoxia alone is probably not sufficiently great to cause rapid respiratory failure.

Since acapnia is not sufficiently marked in 2 of the 3 strychninized animals to alone cause respiratory failure or even when superimposed on the degree of anoxia present (Koehler *et al.* (23)), there must be other physiologic factors which will synergize with anoxia to cause such rapid failure of the respiratory center. It is conceivable that hyperacidemia plus anoxia constitute a more effective means of depressing the respiratory center than anoxia alone. Though blood fixed acids were not determined, hyperacidemia is doubtless present during convulsions since skeletal muscles throughout the body are extremely hyperactive and must be forming acid metabolites which cannot be completely oxidized in the presence of severe anoxia. This contention that the respiratory center is depressed more by hyperacidemia plus anoxia than by anoxia alone is confirmed by the findings of Koehler *et al.* (23).

It has been suggested by Travell and Gold (24) that respiratory arrest is primarily due to fatigue of the respiratory center by excessive bombardment with impulses from the periphery which are normally sub-threshold. An adequate test of this hypothesis was provided in 5 dogs These animals were allowed to convulse for 45–60 minutes while being artificially insufflated The respiratory center still functioned upon discontinuing artificial respiration (fig 13) This proves that excessive bombardment for 45–60 minutes, in the presence of adequate aeration, did not fatigue the respiratory center In order to determine whether asphyxia could cause depression of the respiratory center in these animals, they were subjected to a series of convulsions, without artificial insufflation, lasting 30 seconds At the end of this 30 second period, these dogs made no attempt to respire (fig 13) The asphyxia was partially eliminated by inflating the lungs 7 times After this, arrhythmic respiration started (fig 14) The asphyxia was then completely eliminated by maintaining artificial respiration for 5 minutes (fig 15) When artificial respiration was again stopped, the animals respired normally even though there were several more convulsive seizures (fig 16)



FIGS 13 16 FEMORAL ARTERIAL PRESSURE PULSES (UPPER) AND RESPIRATORY RATE AND AMPLITUDE (LOWER)

FIG 13 Animal allowed to convulse for 60 minutes then artificial insufflation stopped at S_1 and animal allowed to convulse for 30 seconds At I artificial respiration by inflating lungs 7 times then discontinued at S_2

FIG 14 One minute allowed to elapse between this and previous tracing No artificial insufflation

FIG 15
No artificial
tinued for 5 minutes

FIG 16 Artificial insufflation stopped before record taken Animal respired normally even during convulsions

All of these data indicate that anoxemia and hyperacidemia may be more important factors than fatigue by bombardment with sensory impulses in causing fatal respiratory center paralysis during strychnine convulsions

SUMMARY AND CONCLUSIONS

1. Convulsant doses of strychnine in the unanesthetized and narcotized dog and cat do not change the blood pressure until either the nervous or the skeletal muscular component of the convulsion is present.

2. During strychnine convulsions there is an *initial passive rise* in arterial pressure which is primarily the result of an increase in the extra-vascular pressures in the abdominal cavity and skeletal muscles. This is accompanied by: a) extensive activity of the sympathetic and parasympathetic nervous systems tending to cause vasoconstriction and cardiac slowing, *respectively, and* b) *vasodilation* following the excessive skeletal muscular activity.

3. These effects may tend to counterbalance each other or may appear in sequence and produce marked cardiovascular changes and stresses.

4. Asphyxia is the chief causative factor in respiratory depression resulting from strychnine convulsions.

This work was aided by a grant from the Josiah Macy Jr. Foundation. We are indebted to Merck and Company, Rahway for a generous supply of beta-erythroidin hydrochloride and to Dr. Elkin Vogt of Atlanta, Georgia for assistance rendered during some of the early experiments.

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EFFECT OF COCAINE ON THE INACTIVATION OF EPINEPHRINE AND SYMPATHIN

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It is known that cocaine potentiates the effect of stimulation of the sympathetic nervous system and of injected epinephrine There are several possible mechanisms 1) Increased liberation of sympathin during stimulation of the nervous element (suggested by Burn (1) and Jang (2), questioned by Clark and Raventos (3)) 2) Decreased inactivation of the administered epinephrine (4) or sympathin 3) Increased sensitivity of the receptor organ to epinephrine or sympathin (3–15)

While the present work was in progress, a paper appeared by Lawrence, Morton and Tainter (4), showing that cocaine interferes with the inactivation of epinephrine In the results of the present study there are data confirming their findings and further evidence indicating that cocaine also interferes with the inactivation of sympathin

Epinephrine The experiments with epinephrine were performed on the Laewen Trendelenburg (16, 17) preparation of the frog, the perfused rabbit ear, and the perfused rabbit leg

In the Laewen Trendelenburg preparation, frogs were perfused with Ringer's solution through the abdominal aorta and the perfusate collected from the vein of the abdominal wall 0 5 cc of a solution of $1/10^6$ epinephrine was perfused before and again after the preparation was perfused with Ringer's solution containing 0 5 mgm cocaine HCl per 100 cc The rate of inflow was determined by a drop recorder in the inflow circuit, and was kept constant at about 20 drops per minute by adjustment of the pressure whenever a change in the inflow rate took place The perfusate was collected from the vein of the abdominal wall in a tube containing ascorbic acid to prevent oxidation The perfusate was tested by perfusion through a second Laewen Trendelenburg preparation or a frog heart These test objects were cocainized by perfusion with Ringer's solution containing 2 mgm of cocaine HCl per 100 cc This amount of cocaine was sufficiently large to prevent any further action by the cocaine content of the fluid which was to be tested The perfusion rate of the test object was kept constant as described above The solutions to be tested were introduced in 0 5 cc amounts into the inflow circuit over a period of about one minute through a device which prevented the addition of this fluid from changing the rate of inflow in the perfusion circuit The rate of the outflow during the test perfusion was recorded with a drop recorder In the experiments in which the perfused frog heart was used as the test object, the beats were recorded on a kymograph by means of an isotonic lever

The quantity of epinephrine present in the perfusates of a preparation perfused with this drug before and after the preparation was treated with cocaine was compared in terms of the degree of vasoconstriction caused in the legs of the test Laewen Trendelenburg preparation and the degree of cardiac acceleration in the perfused heart The results are summarized in table 1 The perfusion rate in the second Laewen Trendelenburg frog or the heart rate after injection of 0 5 cc. perfusate collected from frogs, with and without cocaine, before injection of epinephrine served as the controls and the results are expressed as a percentage of these values. For this and subsequent tables a 30-second period was chosen for the calculation of the changes in output rate and a 60-second period for the changes in heart rate. The perfusate from the cocaine-treated preparation produced a much greater vasoconstriction than the perfusate from the preparation without cocaine. Therefore the inference is drawn that after cocaine less epinephrine was inactivated during the passage through the tissues. In the case of the frog's heart as the test object, the perfusate from the cocaine treated animal produced greater acceleration, allowing the same inference.

In the experiments with the rabbit ear, the isolated organ was perfused through the auricular artery with Locke's solution at 37°C. The preparation was kept in a humid chamber at 35°C. during the whole experiment. In other respects the technic was similar to that described above. A solution of $1/10^5$ epinephrine, 0.5 cc., was perfused through the ear before cocaine and again after perfusion with Locke's solution containing 1 mgm. cocaine HCl per 100 cc. One cc. samples of the output fluid were tested in a similar manner on the two test objects, namely, the Laewen-Trendelenburg frog and the isolated perfused frog heart, both treated with cocaine. The results are shown in table 2. They confirm the experiments of table 1.

TABLE 1

Comparison of the Effects of Epinephrine Perfusates Obtained from Frogs before and after Cocaine

TEST OBJECT	OBSERVATION	EFFECT IN TEST OBJECT OF EPINEPHRINE PERFUSATE EXPRESSED IN PER CENT OF VALUE BEFORE EPINEPHRINE	
		Obtained before cocaine	Obtained after cocaine
		Mean (26 expts.)	
Laewen-Trendelenburg frog	Maximum slowing	65 ±3.0*	38 ±2.9
	Total decrease in volume	76 ±4.2	32 ±5.1
Isolated frog heart	Greatest increase in rate	143 ±5.5	201 ±16.6
	Total increase in number of beats	111 ±3.2	140 ±3.9

* Standard error of mean.

A third series of experiments was carried out in which the epinephrine was perfused through one leg of a rabbit and the perfusate tested on the second leg of the same animal. The femoral artery was cannulated and perfused with Locke's solution at 37°C. A ligature was then drawn around the leg cutting off all but the femoral artery and femoral vein. The limb was amputated at the hip and placed in a moist chamber maintained at a temperature of 35°C. The perfusate was collected from a cannula in the femoral vein. The second leg was prepared in the same way and perfused with a solution of cocaine containing 2 mgm. per 100 cc. The remainder of the experiment concerning the introduction of epinephrine (0.5 cc. of a $1/10^5$ solution) into the first leg before and after the treatment with cocaine (1 mgm. per 100 cc.), the measurement of the output, and the manner of introducing samples (1 cc.) into the second or test leg were carried out as already described. The results are shown in table 3. They also confirm the previous experiments.

Sympathin. The rabbit ear was prepared for perfusion and the stimulation of the postganglionic fibers from the superior cervical ganglion was carried out according to the method of Gaddum and Kwiatkowski (18), except that the rate of input was lower. The nerve was stimulated by a tetanizing current from an induction coil for periods of 8 seconds. The perfusate was collected from the great auricular vein. One cc. samples were injected into the femoral vein of a cat under pentobarbital anesthesia after the animal was given 3 mgm. of cocaine HCl and 1 mgm. of atropine sulfate per kg. by intravenous injection. The mean blood pressure changes were recorded from the carotid artery with a mercury manometer on a smoked drum. The samples of perfusate were collected under four conditions: before and after sympathetic stimulation, and before and after injection with cocaine.

TABLE 2

Comparison of the Effects of Epinephrine Perfusates Obtained from the Rabbit's Ear before and after Cocaine

TEST OBJECT	OBSERVATION	EFFECT IN TEST OBJECT OF EPINEPHRINE PERFUSATE EXPRESSED IN PER CENT OF VALUE BEFORE EPINEPHRINE	
		Obtained before cocaine	Obtained after cocaine
		Mean (20 expts.)	
Laewen-Trendelenburg frog	Maximum slowing	72 ±4.8	40 ±4.0
	Total decrease in volume	80 ±4.0	30 ±4.9
Isolated frog heart	Greatest increase in rate	140 ±3.7	198 ±6.4
	Total increase in number of beats	117 ±3.9	142 ±4.7

TABLE 3

Comparison of the Effects of Epinephrine Perfusates Obtained from Rabbit's Leg before and after Cocaine

TEST OBJECT	OBSERVATION	EFFECT IN TEST OBJECT OF EPINEPHRINE PERFUSATE EXPRESSED IN PER CENT OF VALUE BEFORE EPINEPHRINE	
		Obtained before cocaine	Obtained after cocaine
		Mean (10 expts.)	
Rabbit leg	Maximum slowing	68 ±3.8	36 ±4.2
	Total decrease in volume	80 ±2.9	52 ±5.0

After cocaine the outflow from the stimulated rabbit ear decreased to a mean of 85 per cent of the outflow before cocaine (observations made on 10 cats, the standard error of the mean being ± 1.3). As is the general belief, therefore, cocaine intensifies the vasoconstrictor effect of electrical stimulation of the sympathetic nerves. The outflow from the preparations before cocaine increased the blood pressure of the cat by an average of 10%, while those from the cocaine treated preparations produced a 20% increase. The differences are much more striking when expressed as the percentage difference between the blood pressure effect of non-cocainized and cocainized samples for each rabbit. When the average of those differences is taken, cocaine is found to cause an effect which is 87% greater than that obtained before cocaine (table 4). This result is in harmony with the results obtained with epinephrine and indicates that cocaine interferes with the tissue inactivation of sympathin as well as epinephrine.

TABLE 4

Effect of Cocaine on the Sympathin Content of Perfusion Fluid from Stimulated Rabbit Ear

Changes in the mean blood pressure of the cat in mm. Hg after injection of 1 cc. of perfusate from rabbit ear

OBTAINED BEFORE COCAINE				OBTAINED AFTER COCAINE			
Before stimulation		After stimulation		Before stimulation		After stimulation	
Control	After dose	Control	After dose	Control	After dose	Control	After dose
100	100	99	108	94	95	95	115
117	118	118	126	101	101	100	125
88	88	88	100	85	85	84	102
84	84	83	90	89	90	90	106
100	100	99	120	110	110	110	138
96	97	97	108	93	93	92	110
86	87	87	100	90	90	88	101
77	77	76	90	85	85	84	100
92	92	92	100	89	90	90	111
120	120	119	128	121	121	120	133
Average increase followed by standard error of mean							
0.3 ±0.05		10.2 ±0.74		0.3 ±0.05		17.8 ±1.46	

SUMMARY AND CONCLUSIONS

1. A solution of epinephrine was perfused through the Laewen-Trendelenburg frog preparation, the rabbit ear, and the rabbit leg, before and after treatment with cocaine. The perfusates were tested for their vasoconstrictor action on the perfused Laewen-Trendelenburg preparation and the rabbit leg, and for their effect in accelerating the rate of the isolated perfused frog's heart.

2. The results show that after cocaine the perfusate containing epinephrine exerts a greater effect.

3. The conclusion is drawn that cocaine interferes with the inactivation of epinephrine in the tissues.

4. The postganglionic fibers from the superior cervical ganglion were stimulated in the rabbit ear preparation of Gaddum and Kwiatkowski. The perfusate from the ear was tested on the blood pressure of the cat. The results of

these experiments show that the fluid passing through the tissue when the sympathetic nerve is stimulated has a greater vasopressor action than the perfusate collected under the same conditions in the absence of cocaine

5 The conclusion is drawn that cocaine also interferes with the inactivation of sympathin

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THE INFLUENCE OF LOW CONCENTRATIONS OF COCAINE ON THE METABOLISM OF PHENOL

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The inhibition of the esterification of phenols by cocaine *in vivo* (1), the inhibition of phenol sulfur esterase *in vitro* (2), and the fact that cocaine potentiates the action of epinephrine may lend an indirect support to the theory of Richter (3, 4) that epinephrine is inactivated partly by esterification of the phenol ring. In the present communication the effect of low concentrations of cocaine on the conjugation of phenol will be described.

METHOD. Cats were anesthetized with pentobarbital. To avoid inactivation of phenols in the intestine (5, 6) this organ was excised. The blood pressure was registered by a cannula inserted in the carotid artery leading to a mercury manometer. The blood pressure was kept nearly constant and satisfactory urine flow insured by infusion of Locke's solution when necessary. Capillary glass cannulae were inserted into the two ureters and the urine was collected separately from each kidney in samples eliminated in 30-minute periods throughout the experiment. Five minutes after the start of each collection a sample of blood was taken from the femoral artery for the purpose of determining the phenol content of blood cleared by the urine eliminated during the corresponding 30-minute period. After a few samples were collected the cat was injected through the femoral vein with one of the following substances: *1*) cocaine, *2*) phenol, *3*) phenol and cocaine. The rate of injection was constant so that 10 mgm. per kg. of phenol or 4 mgm. per kg. of cocaine were introduced in a three-hour period. In the case of phenol and cocaine the infusion of cocaine was started one hour after the beginning of the infusion of phenol. In another series of experiments the entire dose of phenol, 6 mgm. per kg., was given as a single injection into the femoral vein and the blood and urine samples were collected as described above. The blood and urine of cats treated in exactly the same way and for the same length of time as the injected animals were used as controls.

The free and conjugated phenol content of the blood and urine samples were determined with the method of Theis and Benedict (7, 8, 9). The color changes produced by the phenol present were determined by a photoelectric colorimeter. The changes in the phenol content of the blood and urine are summarized in the tables. The values are expressed as a percentage of the phenol content of the samples collected shortly before the first injection. The values given in the tables are calculated on the basis of the phenol content of blood (mgm. per 100 cc.) and urine (amount eliminated in 30-minute periods), since these values give a better indication of phenol metabolism than does the clearance.

RESULTS. Cats eliminated in the urine an average of 0.007 mgm. free and 0.0047 mgm. conjugated phenol per kg. of body weight per minute. The blood contained on the average 1.8 mgm. free and 0.19 mgm. conjugated phenol per 100 cc. The phenol content of blood and urine of the control cats remained nearly constant over a period of three hours (table 1).

After injection of a single large dose of phenol the blood phenol immediately reached a peak and then decreased gradually to the original level over a period of about two hours (see also 5, 6, 10, 11, 12, 13, 14, 15). The phenol content of blood decreased partly by elimination through the kidney and partly by diffusion to the tissues The conjugated phenol soon appeared in the blood Whether the conjugation occurs partly in the blood or entirely in the tissues is not known The free and conjugated phenol content of the urine rose Conjugated phenol was eliminated and the concentration in the blood returned soon to its original value (table 1)

Experiments in which the injection of phenol was continuous show that the free and conjugated phenol content of blood and urine increased gradually (figure 1A, table 1) As the infusion was continued the excretion rate reached a maximum after which the conjugated phenol content of blood rose suddenly

TABLE 1

Effect of injected phenol and cocaine on the phenol content of blood and urine

EXPERIMENTAL PROCEDURE	NO OF EXPTS	MEAN FREE PHENOL CONTENT OF BLOOD IN PER CENT OF CONTROL* AT 30 MINUTE INTERVALS FROM START OF INJECTION							MEAN FREE PHENOL CONTENT OF URINE ELIMINATED IN PER CENT OF CONTROL* IN SUCCESSIVE 30 MINUTE PERIODS FROM BEGINNING OF INJECTION						
		0 min	30 min	60 min	90 min	120 min	150 min	180 min	0 min	30 min	60 min	90 min	120 min	150 min	180 min
Control	6	100	100	99	100	99	98	96	100	102	98	97	98	95	97
Phenol injected	10	100	223	217	184	170	108	108	100	187	191	191	205	202	200
Phenol infused	12	100	118	133	148 (111)†	172 (129)†	180 (135)†	190 (143)†	100	125	132	145 (110)†	164 (124)†	190 (144)†	202 (153)†
Phenol and cocaine infused	16	100†	118	130	145	160	180	200	100†	126	135	142	166	180	200
Cocaine infused	6	100	102	105	109	110	111	110	100	110	102	109	113	115	118
		MEAN CONJUGATED PHENOL CONTENT OF BLOOD IN PER CENT OF CONTROL*							MEAN CONJUGATED PHENOL CONTENT OF URINE IN PER CENT OF CONTROL*						
Control	6	100	95	100	102	98	97	96	100	106	96	100	97	95	98
Phenol injected	10	100	272	240	246	208	195	208	100	332	369	413	497	524	524
Phenol infused	12	100	12	290	326	358	444	530	100	237	494	4 8	499	534	544
Phenol and cocaine infused	16	100†	109	103	100	94	85	80	100†	168	151	124	113	99	100
Cocaine infused	6	100	96	90	86	82	80	80	100	95	92	89	87	84	70

* The standard error of mean for each value was less than ±10 per cent

† Expressed as a percentage of the value after 60 minutes of infusion with phenol

Injection of cocaine did not significantly change the free phenol content of blood and urine Changes in the conjugated phenol content were too small to be conclusive indication of a modification of the enzymatic processes (table 1)

When cocaine was introduced during phenol perfusion the conjugated phenol in the blood did not increase further but, on the contrary, decreased The kidneys continued to clear the blood of its conjugated phenol in the presence of a small amount of cocaine Later in the course of the experiment the free phenol content of blood increased somewhat (figure 1B, table 1)

DISCUSSION Cocaine, even in very low concentrations, inhibits the enzymatic processes involved in the conjugation of phenols in the living animal This inhibition is manifested in cats continuously injected with phenol by a sudden fall of the conjugated phenol in the blood, a slow decrease of the conjugated phenol excretion in the urine and a rapid rise in the free phenol content of blood and urine. This contrasts with the increase of the conjugated phenol content of blood and urine and the very slow increase of free phenol in the absence of cocaine. Cocaine alone did not change significantly the elimination of phenol. This is probably due to the nonspecificity of the method suggesting that the values obtained for the normal urine are mainly due to another substance (see 16 and 17).

These results are in agreement with the results obtained in metabolic experiments (1) and with enzyme experiments *in vitro* (2). They lend an indirect support to the theory of Richter (3, 4) that the body may dispose of epinephrine partly by esterification of the phenol ring.

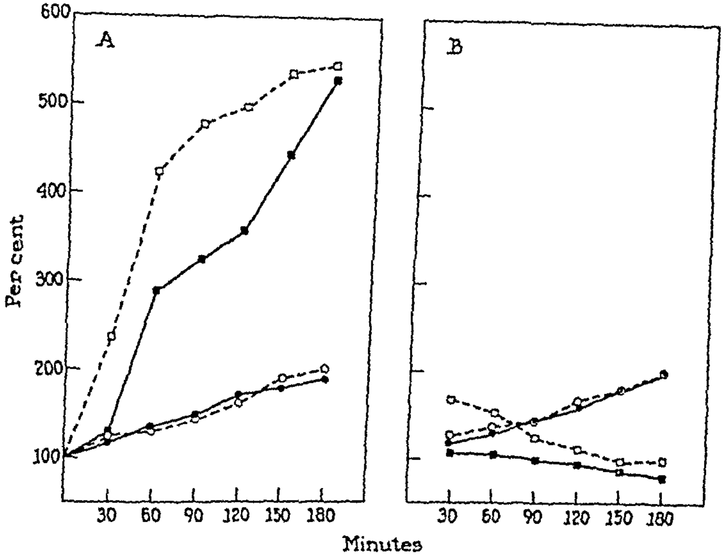

Fig. 1. Comparison of the Phenol Content of Blood and Urine during Continuous Injection of (*A*) Phenol and (*B*) Phenol and Cocaine

The per cent changes of the phenol content is plotted against the time in minutes. Each point represents the mean value of from 6 to 16 experiments.

Shaded circles: Free phenol content of blood. Shaded squares: Conjugated phenol content of blood. Open circles: Free phenol content of urine. Open squares: Conjugated phenol content of urine.

SUMMARY

1. The effect of slow cocaine infusion on the phenol metabolism of living cats was studied by determination of the free and conjugated phenol content of the blood and the corresponding urine samples.

2. A single large injection of phenol results in an increase in the free and conjugated phenol in the blood and urine lasting a few hours.

3. A continuous infusion of phenol produces a gradual rise of the free and conjugated phenol in the blood and urine. As the infusion is continued, the excretion

rate reaches a maximum and the conjugated phenol content of the blood rises suddenly.

4. Infusion of cocaine in a phenol infused cat inhibits the process of conjugation resulting in a decrease of the conjugated phenol content of blood and urine.

5 These results indicate that cocaine, even in low concentrations, inhibits the enzymatic processes involved in the conjugation of phenol in the living animal. They also lend an indirect support to the theory of Richter that epinephrine may be inactivated partly by esterification of the phenol ring

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THE COMPARATIVE TOXICITY OF CHLORAL ALCOHOLATE AND CHLORAL HYDRATE

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The familiar terms "knock-out drops" and "Mickey Finn" characterize the potent hypnotic effect of solutions containing both chloral hydrate and alcohol. Statements (1 to 6) frequently have appeared advising against the use of these drugs in the same preparation. The reason usually given for these warnings is that a toxic compound is formed, *e.g.*, "the chloral alcoholate formed" (1), "the formation of a toxic agent" (2), and "chloral alcoholate . . . a powerful and rapidly acting cerebral depressant" (3). No data were found in the literature to support the implication of these statements that the compound formed is more toxic than chloral hydrate. Indeed, adequate data to justify the claim that a toxic compound is formed at all appear to be lacking. Hargreaves (4) has shown that, when liquid preparations contain alcohol, chloral hydrate and an alkali bromide, a separation incompatibility may occur due to the "salting out" of an oily layer which is said to contain chloral, chloral alcoholate and alcohol. Since all of the chloral may be found in the oily layer, the danger of overdosage is present when the preparation is used without proper shaking. This incompatibility is justification for warnings against such mixtures but gives no information on the toxicity of preparations in which the separation does not occur.

Chloral alcoholate is available in crystalline form, but it has been claimed that in aqueous solution it is converted to chloral hydrate (7). If this is true, it follows *à priori* that there is practically no possibility of systemic action from chloral alcoholate, as such, when taken by mouth. A strongly alcoholic vehicle for the chloral alcoholate would not prevent the change to chloral hydrate *in vivo*. For, in addition to the decrease in alcoholic strength resulting from dilution by alimentary fluids, the drug would be transported in an essentially aqueous medium (blood plasma) before it reached the central nervous system. Certainly the blood concentration of alcohol or chloral hydrate during the most profound depression by either of these drugs (less than 1% in either case) does not alter the aqueous properties of plasma. By the same reasoning, therefore, any chloral alcoholate formed *in vitro* by interaction of chloral hydrate and alcohol could not reach the nervous system as chloral alcoholate. Acceptance of this reasoning is tantamount to repudiation of chloral alcoholate as the toxic agent of "knock-out drops".

However, adequate proof that chloral alcoholate in aqueous solution becomes chloral hydrate is unavailable. Apparently this is due to the lack of specific methods for determining chloral alcoholate or chloral hydrate in solutions containing both of these drugs The identification of chloral alcoholate in oily liquids from which water has been removed (4) does not solve the problem Regardless of probabilities, the possibility remains that chloral alcoholate, as such, may reach the central nervous system It appeared, therefore, worth while to investigate the toxicity of solutions obtained by dissolving chloral alcoholate in water

EXPERIMENTAL A comparative study of the hypnotic potencies, acute toxicities, and chronic toxicities of solutions made by dissolving 4 grams of crystalline chloral alcoholate[1] or chloral hydrate in 100 cc of distilled water was carried out on rats, and a few additional observations of the acute toxicities of these solutions were made on rabbits cats and dogs All administrations were made by stomach tube to animals from which food but not water, had been withheld 16 hours The dosages of the solutions were proportional to body weights and are reported in terms of the weights of the drugs which were dissolved in the volumes administered Four per cent solutions were sufficiently dilute to avoid irritation and vomiting in most cases and yet not so dilute as to require excessive volumes which would have overdistended the stomach when the larger doses were administered Alcoholic solutions of chloral alcoholate were not used for the reasons given above and because the comparisons of these experiments would have been vitiated by the introduction of additional depression from the alcohol alone

Hypnotic potencies were compared by observing the duration of hypnosis (sleep) in white rats which had received the chloral alcoholate or chloral hydrate solution in amounts equivalent to 0 4 gram of drug per kgm Each solution was administered to 18 rats (table 1) and all tests were concurrent The period of hypnosis was arbitrarily taken as the elapsed time between the moments (*a*) when voluntary movements ceased and the rat failed to right itself when placed on its side and (*b*) when the rat voluntarily sat erect again

Acute toxicities were determined by recording deaths in groups of white rats which received the chloral alcoholate or chloral hydrate solution in graded doses ranging from 0 5 to 1 3 gram of drug per kgm (fig 1) More than 200 white rats weighing 125 to 250 grams each were used Observations (Table 2) were also made on 36 rabbits (1 to 2 6 kgm), 9 cats (1 2 to 4 4 kgm), and 16 dogs (1 2 to 14 4 kgm) These studies were carried out over a period of several years as groups of animals became available Whenever a group was tested some of the animals received the chloral alcoholate solution while others received the chloral hydrate solution In a few instances those animals which had received the smaller doses were used a second time but never without a rest period of at least 7 days between tests to avoid the accumulation which may occur if tests are repeated within 4 days (8)

The chronic toxicities of these solutions were determined by repeating the administration of a hypnotic dose (0 4 grams of drug per kgm) twice weekly to the same group of rats for six weeks and then examining each rat at autopsy Two series of studies were carried out in different laboratories Chloral alcoholate solution was given to a total of 26 white rats and chloral hydrate solution to 12 Concurrently control groups totaling 20 rats were observed Each control rat received a comparable volume of tap water by stomach tube each time that the drugs were administered Following the 12 administrations all rats were killed and examined for evidence of gross pathologic lesions The heart, liver, kidneys stomach aorta lungs and spleen from 16 rats on chloral alcoholate solution 4 on chloral hydrate solution and 9 on control (distilled water) were fixed in 10% formaldehyde solution and slides of these tissues were prepared for microscopic examination

RESULTS The hypnotic potencies of the chloral alcoholate and chloral hydrate solutions are compared in the summarized data of table 1 The averages show practically no difference of effect between the solutions. Following either solution, the hypnosis time of the individual rat tended to be inversely proportional to its body weight, but moderate variation in the hypnosis times of rats of the same weight was observed. No differences of effect attributable to sex were apparent, and judging by hypnosis times, tolerance was not developed.

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[1] Obtained from Eastman Kodak Co

All data of acute toxicity in rats are represented in the toxicity curves of fig. 1. An LD_{50} of 0.88 gram of drug per kgm. for the chloral alcoholate solution and 0.80 gram of drug per kgm. for the chloral hydrate solution indicates a slightly higher toxicity for the latter. Acute toxicity was the same for both sexes.

TABLE 1

Comparative hypnotic effect of aqueous solutions of chloral alcoholate and chloral hydrate in rats

DRUG, 0.4 GRAM PER KGM.	RATS		WEIGHT		DURATION OF SLEEP	
	Male	Female	Range	Average	Range	Average
4 per cent solution	*number*		*grams*		*minutes*	
Chloral alcoholate..........	7	11	168–246	202.3	120–215	157.8
Chloral hydrate	5	13	160–242	201.7	90–210	156.0

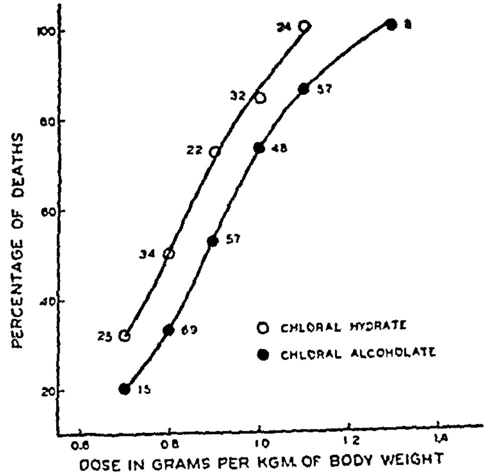

FIG. 1. THE ACUTE TOXICITY OF 4% AQUEOUS SOLUTIONS OF CHLORAL ALCOHOLATE OR CHLORAL HYDRATE ADMINISTERED TO RATS BY STOMACH TUBE

The number of rats receiving each dose is indicated by the number opposite each point on the curves

The acute toxicities of the limited studies on rabbits, cats and dogs are given in table 2. These data, too, indicate that the MLD of the chloral alcoholate solution is higher than that of the chloral hydrate solution. The protocols of these experiments, with respect to hypnosis times, etc., were too extensive for inclusion in this report, but they were of considerable aid in determining the relative effects of the solutions. Cats showed considerably greater susceptibility to both solutions than did any of the other species. In contrast to approximately 2 to 2½ hours of sleep in rabbits or dogs following a dose equivalent to 0.5 grams of either drug per kgm., cats showed the following effects. A dose of chloral alcoholate solution equivalent to 0.5 grams of drug per kgm. was followed by (*a*) vomiting in two which recovered the following day, (*b*) 4½ days of comatose sleep in a third which then recovered, and (*c*) 5 days of coma in two which, apparently *in extremis*, were then killed. A smaller dose of the chloral hydrate solution, equivalent to 0.4 grams of drug per kgm., was followed by (*a*) 36 hours of comatose sleep in two with full recovery in 3 days, (*b*) 48 hours of comatose sleep in one with recovery in 4 days, and (*c*) death of one during the 4th day of coma.

TABLE 2

Acute toxicities of 4 per cent aqueous solutions of chloral alcoholate or chloral hydrate administered by stomach tube to rabbits, dogs, and cats

The upper number of each pair indicates the number of animals receiving the corresponding dose; the lower number indicates the number of fatalities, due to the drug, in the corresponding group.

DOSE GRAMS/KGM	CHLORAL ALCOHOLATE			CHLORAL HYDRATE		
	Rabbits	Dogs	Cats	Rabbits	Dogs	Cats
0.50	11 0	8 0	5 2		3 0	4* 1
1.00	10 0	7 0		8 1	3 1	
1.10	11 2	1 0			6 5	
1.20	12 2	4 1		12 4		
1.30	8 1	4 3		4 4		
1.40	6 3	1 1		6 5		
1.50	4 4			3 3		

* This dosage was 0.4 gram/kgm., see text.

All rats of the chronic toxicity series gained weight during the six weeks of study. On gross examination all organs and tissues appeared to be normal. The microscopic examination of approximately 200 separate tissues from the 29 rats revealed no pathology, except one case of acute respiratory infection.

DISCUSSION. Each phase of this investigation demonstrated that the solution of chloral alcoholate was *not* more toxic than that of chloral hydrate. The qualitative responses to these solutions, observed under the same conditions, were indistinguishable. The hypnosis studies shown in Table 1 were similar to previous experiments (9) in which the differences between hypnosis times never exceeded 10%, but in which the longer periods usually followed chloral hydrate. In general, then, these data show that there is no practical difference between the hypnotic effects of the chloral alcoholate and the chloral hydrate solutions. The acute toxicity studies in rats consistently showed *that the solution of chloral alcoholate* was slightly less toxic than that of chloral hydrate. Comparing the molecular weights of these drugs (alcoholate 193.43, hydrate 165.39), it appears that the depressant effects were proportional to the number of chloral groups administered. The acute toxicity studies in the other species follow the same pattern, *i.e.*, slightly less toxic effects from the solution of chloral alcoholate. Finally, the chronic studies revealed no basis for regarding the solution of chloral alcoholate as especially toxic. Thus, neither in the literature nor in these studies could evidence be obtained to support a widely held belief that chloral alcoholate is responsible for a particularly potent depression of the central nervous system. Additional studies are now indicated to determine whether the "knock-out" effect of preparations containing both alcohol and chloral hydrate is due to (*a*) overdosage, (*b*) facilitated absorption of chloral hydrate in the presence of alcohol, (*c*) simple summation of the depression from each drug, or (*d*) potentiation of depression resulting from the concurrent use of chloral hydrate and alcohol.

This report apparently not only records the first toxicity studies of chloral alcoholate, but also provides the first adequate determination of the LD_{50} of chloral hydrate solutions administered orally to rats. Griffith and Farris (10) calculated an LD_{50} for rats from data (11) which, although presumably the only data available, were inadequate and obviously not intended for such purpose.

The delayed recovery of cats following the administration of solutions of chloral hydrate or chloral alcoholate suggests that the process of detoxification or excretion of these drugs is extremely slow in this species, but no data are available to confirm this. The fatal dose of chloral hydrate given by stomach tube to cats had been reported as 0.44 grams per kgm. by Sollmann and Hatcher (12). The limited data of the present report are in accord with this figure.

SUMMARY

The hypnotic potencies, acute toxicities, and chronic toxicities of 4% aqueous solutions of chloral alcoholate and chloral hydrate administered to rats by stomach tube have been compared. Additional limited observations of acute toxicity were made also on rabbits, cats, and dogs.

The qualitative responses to these solutions were indistinguishable, but quantitatively, in all species, the solution of chloral alcoholate was slightly *weaker* in its depressant action than that of chloral hydrate. In rats, according to this method of comparison, the LD_{50} for chloral alcoholate was 0.88 grams per kgm.; for chloral hydrate 0.80 grams per kgm. Cats were observed to be markedly more susceptible to the depressant action of these solutions than any of the other species.

On the basis of available evidence and the data of this study there appears to be no support for the widespread impression that solutions containing alcohol and chloral hydrate are particularly depressant because of the formation of chloral alcoholate as an especially toxic compound

Acknowledgement To Dr Roy R Kracke, Emory University, Georgia, and to Dr Arthur W Wright, Albany, New York, I express my appreciation for the preparation and examination of the numerous slides in the chronic toxicity series of this study

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THE ABSORPTION OF QUININE SULFATE AND QUININE DIHYDROCHLORIDE FROM ISOLATED INTESTINAL LOOPS OF DOGS

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The mechanism of the therapeutic action of quinine as an anti-malarial has never been known. Whether this action is a direct result of the attainment of optimum levels of unchanged quinine in the blood stream or whether the schizontocidal action results from the presence of some metabolic decomposition product of the drug has been and still is a matter for disagreement. In either case, however, the first pertinent factor to be considered is the efficiency with which the dose is absorbed. Absorption studies on quinine salts have, in the past, taken the form of administration of the drug by mouth followed by periodic analysis of blood by various methods. It is obvious, however, that following its administration, the level of quinine in the blood stream is a result not only of the rate of absorption from the intestine, but also of the rates of its removal into the urine and of its metabolic destruction. More direct measurements of the rate of its disappearance from the intestine are therefore desirable. Such measurements may take either of two courses: the whole intestinal tract of the animal may be employed as in the Cori (1) technique, or an isolated segment of the intestine may be employed as described by Johnston (2). The former method gives values representative of the absorptive ability of the whole normal animal but labors under the disadvantage of requiring the sacrifice of each animal as a part of the procedure. Strictly comparable results are less possible therefore than in the use of isolated intestinal loops. Although such loops represent only a fraction of the absorbing surface of the animal, they make possible more accurate comparisons between different compounds since repeated determinations can be made on the same animal and there is no possibility of fecal loss.

In the present paper we wish to report on the relative rates of absorption of quinine sulfate and quinine dihydrochloride from such a loop. Because of its insolubility, the relatively much more common and inexpensive sulfate would be expected to be less rapidly absorbed than the dihydrochloride. On the other hand, the same argument appears not to hold when applied to a natural but relatively insoluble amino acid such as cystine. Measurements reported by Andrews, Johnston and Andrews (3) of the relative rates of absorption of cystine in the insoluble isoelectric condition and as the very soluble sodium salt gave practically identical results. It is possible however that a distinction should be drawn between a natural product of protein digestion and a comparatively "foreign body" such as quinine.

METHODS AND RESULTS. Dogs with jejunal loops were prepared for us by the courtesy of Dr. C. G. Johnston, of the School of Medicine of Wayne University, by the method described by him (2). One such dog, a spaniel weighing

13.85 kilos, was used for the measurements described below. In order not to fatigue the animal, intervals of at least three days were allowed to elapse between each absorption experiment. At all times, the dog was kept on a normal animal house diet.

The duration of such absorption experiments must necessarily be such that not all of the substance being tested is absorbed. A number of preliminary trials are therefore necessary to establish a proper period for each substance investigated. It is, moreover, necessary to determine whether quantitative recovery of the introduced substance can be obtained when the latter is washed out of the loop immediately after its introduction. Careful determination of this point is especially necessary in cases where, as with quinine sulfate, complete solution of the administered drug cannot be attained.

In all experiments, whether tests of absorption speed or of recovery of the drug, the loop was first washed free of accumulated mucus by means of physiological saline solution. The rubber catheter with balloons was then introduced, the balloons inflated and after further washing the charge of the drug was introduced from a 50 ml. syringe. In case of quinine dihydrochloride the dose was dissolved in water, in case of the sulfate it was suspended. In both cases all traces of the quinine salt were washed into the loop by successive small amounts of warm water

After removal of the quinine salt from the loop and thorough washing of the latter, the combined solution and washings were made up to a definite volume (1000 ml.) and at least four aliquots were analyzed for quinine by the method by Kyker, Webb and Andrews (4).

Preliminary attempts to remove the introduced salt at once and quantitatively gave very incomplete recoveries, particularly in the case of the sulfate The recovery figures usually ranged from 65 to 75% of the drug. In the case of the dihydrochloride, subsequent work indicated that these low recoveries were largely due to the very rapid absorption of the drug. A more speedy technique raised these figures to values varying from 90 to 98%. However, in the case of quinine sulfate the removal of portions of the solid salt, surrounded by clots of mucus, indicated that the difficulty was largely mechanical.

More quantitative recoveries of quinine sulfate were finally obtained by taking advantage of the increased solubility of quinine sulfate in 0.9% sodium chloride solution. The rather surprising increase in the solubility of quinine sulfate in increasing concentrations of sodium chloride appears not to have been previously reported in the literature. A number of measurements were therefore made of the solubility of quinine sulfate in sodium chloride solutions at 40° ± 0.01°C.

For the purpose of these measurements, an excess of pure recrystallized quinine sulfate was suspended in the sodium chloride solution in a flask, immersed in the constant temperature bath and occasionally agitated. At intervals, aliquots of the clear liquid were withdrawn and the attainment of equilibrium between solid and liquid phase determined by constancy of the optical activity of the solution When this point was reached, measured volumes of the solution were weighed and used for the determination of the quinine content (4). The data are recorded in table 1. This solubility curve is of interest in that it reaches a maximum at about 2 to 4% sodium chloride after which it decreases rapidly.

Taking advantage of this enhanced solubility in physiological saline, the latter was used for washing out the loop. A series of such experiments gave recoveries of 93, 90, 86, 93 and 98%. It would seem reasonable to suppose that in the case of the dihydrochloride solubility considerations do not enter and that rapid technique alone contributes toward successful recoveries. With quinine sulfate, however, the question of solubility as well as of moderate speed of absorption no doubt influences recoveries unless the sodium chloride technique is used. The very rapid absorption of the dihydrochloride has been commented on by Kaiser (5) who was able to demonstrate quinine in the blood of dogs eight minutes after giving the dose by mouth. He also reported similar findings for quinine sulfate.

For all measurements herein reported the dose of the quinine salt used was that corresponding to 0.020 grams quinine sulfate per kilo of body weight, an amount comparable to normal human dosage. This corresponds to 0.0174 gram free base per kilo or to 240.6 mgm. free base for the whole animal. Amounts of either sulfate or dihydrochloride corresponding to the above amount of free base were therefore used for all experiments.

TABLE 1

Solubility of quinine sulfate ($Q_2H_2SO_4$) in increasing concentrations of sodium chloride at 40°C. (both concentrations expressed as grams per 100 grams solution)

CONC. OF NaCl	CONC. OF QUININE SULFATE
0	0.2398
0.6154	0.4585
1.9913	0.4968
4.9392	0.3162
9.4688	0.1532

While the chief object of our experiments was to measure the relative rates of disappearance of quinine sulfate and quinine dihydrochloride from the intestinal loops, the levels of quinine attained in the blood stream and excreted in the urine were also followed. Before each absorption experiment, a normal blood sample was taken, usually from the right jugular vein, and the urinary bladder was emptied by catheterization. These normal samples, although from the unmedicated animal, were used for the usual determination of quinine in order to assure the absence of any substance which might behave like quinine (4). The charge of quinine salt was then introduced and, on completion of the experiment, removed as described above. Immediately after washing of the loop was complete, a sample of blood was taken as before and the total accumulation of urine since taking the normal sample at the beginning of the experiment was removed. The quinine content of both the blood and the urine was then determined by the same method (4) as that used for the washings from the loop. In case of blood and urine samples, two separate extractions were made with triplicate readings on each extraction. All figures reported are the average of all triplicates.

The data obtained from these measurements are recorded in table 2. It will be noted that all figures for quinine concentrations and recoveries are expressed as the free base, regardless of which salt was used. In general, the percentages of the drug absorbed for any given length of experiment are more irregular for the sulfate than for the dihydrochloride. This would be expected in view of the much greater difficulty of washing out in any quantitative way, the particles of solid quinine sulfate, often surrounded by a mucous coating and adhering to the intestinal wall. Based on the solubility of quinine sulfate in 0.9% sodium chloride as reported in table 1, the 300 cc. of this solution used for washing the loop should be much more than necessary to effect complete solution if attainment of equilibrium is not hindered by mucous coatings.

TABLE 2

The absorption of quinine as the sulfate and the hydrochloride from an isolated intestinal loop of a dog weighing 13.85 kg.

All doses of quinine salts contained 240.6 grams free base

LENGTH OF EXPT	QUININE		BLOOD		URINE		
	Recovered	Percentage absorbed	Time from dose to blood collection	Conc. of quinine	Total excretion period	Quinine conc.	Total recovered
			Quinine sulfate				
min.	*mgm.*		*min.*	*mgm./l.*	*min.*	*mgm./l.*	*mgm.*
60	54.5	77.4	72	5.06			
60	72.2	70.0	63	3.01	84	188.6	2.64
60	65.0	73.0	84	4.62	111	180.7	1.45
60	74.7	69.0	68	4.62	96	196.7	2.30
60	73.6	69.4	63	4.71	77	190.9	2.34
			Quinine dihydrochloride				
10	76.9	68.0	13	3.61	30	132.9	1.18
10	83.1	65.5	15	3.14			
10	81.3	66.2	15	3.01	31	140.6	1.20
10	80.1	66.7	14	3.76	33	119.8	1.80
15	37.7	84.3	23	9.05	46	151.1	0.76
30	27.1	88.7	45	4.36	60	123.6	0.87
60	6.9	97.1	85	2.69	105	40.7	1.63
60	16.2	93.3	62	2.03	110	127.5	0.89
115	6.9	97.1			140	128.7	1.80

The much slower rate of absorption of quinine sulfate as compared with the dihydrochloride is obvious. Nearly as much of the latter is absorbed in ten minutes as of the former in sixty. However it should not be concluded that this experimental fact necessarily justifies the assumption of greater antimalarial efficiency on the part of the dihydrochloride. The latter undoubtedly produces a more rapidly reached and perhaps a transiently higher peak of blood quinine concentration but as indicated in table 2 the level of quinine in the blood 60 minutes after administration in the loop has remained nearly twice as high in

THE ABSORPTION RATES OF INSULIN, GLOBIN INSULIN AND PROTAMINE ZINC INSULIN LABELLED WITH RADIOACTIVE IODINE

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Both protamine (1) and globin (2) when combined with insulin and injected subcutaneously result in a less intense but more prolonged hypoglycemic action than is obtained with insulin alone. This fact has been explained by the assumption that the material remains at the site of the injection for a considerable length of time and that it releases insulin slowly. Beecher and Krogh (3) have studied the absorption of insulin and protamine insulin using methylene blue as a tracer. Their experiments suggested that insulin was absorbed several times as rapidly as protamine insulin. Reiner, Keston and Green (4) have described the use of radioactive iodine in measuring the absorption rate of insulin after subcutaneous injection. The determination of the relative rates of absorption of insulin, globin insulin, and protamine zinc insulin labelled with radioactive iodine is the object of this investigation.

EXPERIMENTAL. The insulin used was labelled by the introduction of *p*-azoiodobenzene (containing radioactive iodine) into the insulin molecule. The introduction of iodine in this manner causes no significant change in potency and the insulin azo compound can be obtained in crystalline form if desired (5).

Preparation of Iodoaniline. The methods for micro technique described by Morton (6) were used in the synthesis of *p*-iodoaniline and in subsequent manipulations. Radioactive iodine of 8-day period was prepared by deuteron bombardment of tellurium in a target of cobalt-copper telluride. One hundred micrograms of iodine as potassium iodide were added and the target was dissolved in 6 molar nitric acid. The iodine was distilled into carbon tetrachloride from which it was extracted with a solution of sodium thiosulfate equivalent to the added iodine. The solution containing 100 micrograms of the radioactive iodine was then evaporated to a volume of about 0.10 cc. This solution was then acidified by the addition of hydrochloric acid and the iodine liberated by adding a slight excess of potassium iodate, each reactant being contained in 0.01 cc. The solution was then made alkaline with sodium bicarbonate and one micromol of aniline added. The mixture was stirred intermittently for a half hour, then reacidified with hydrochloric acid, and about half the previous amount of potassium iodate added. It was made alkaline with sodium bicarbonate and stirred intermittently as before. This was repeated a third time to insure the conversion of the major part of the radioactive iodine into *p*-iodoaniline.

Preparation of Insulin-p-azoiodobenzene. The mixture was then acidified with hydrochloric acid and the *p*-iodoaniline diazotized in an ice bath by the slow addition of sodium nitrite. To this solution was added 10 mgm. of insulin, equivalent to 200 I. U., dissolved in the minimum amount of N/10 hydrochloric acid, and the pH was adjusted to between 8 and 9 by the addition of sodium hydroxide and disodium phosphate. The solution was allowed to stand for one hour in the ice bath to insure complete coupling. The insulin azo dye was then precipitated several times at its isoelectric point in the presence of non radioactive potassium iodide, *p* iodoaniline and *p* iodophenol and was finally dissolved in N/10 hydrochloric acid and diluted to 1 0 cc to give a concentration of 200 I U per cc

Globin Insulin To 0 25 cc of the insulin azo dye solution was added 0 15 mgm of zinc in the form of zinc chloride solution and sufficient globin solution to contain 1 9 mgm of native globin The pH was adjusted to about 3 5 by the addition of N/10 NaOH and the solution diluted to a concentration of 80 I U per cc using distilled water containing sufficient tricresol to give a final concentration of 0 10 per cent This gave a clear yellow solution which contained 0 30 mgm zinc and 3 8 mgm of globin per 100 I U insulin

Protamine Zinc Insulin To 0 375 cc of the insulin azo dye solution were added solutions containing 0 15 mgm of zinc as zinc chloride, 1 5 mgm of protamine as protamine sulfate, 1 5 mgm disodium phosphate and 15 mgm glycerine The pH was then adjusted to about 7 by the addition of dilute sodium hydroxide and the solution diluted to a concentration of 80 I U per cc using distilled water containing sufficient tricresol to give a final concentration of 0 20 per cent The whitish yellow precipitate which formed settled out very slowly on standing leaving a clear colorless supernatant liquid indicating almost complete precipitation of the insulin

Insulin Solution The 0 375 cc of the insulin azo dye solution remaining was diluted to a concentration of 80 I U per cc using distilled water containing sufficient tricresol to give a final concentration of 0 10 per cent This gave a clear yellow solution having a pH of about 3

Absorption Rate Determinations Albino rabbits weighing from 2 5–3 5 kgm were starved for 22 hours previous to and during the experiments The rabbits were divided into three groups and a blood sample for sugar determination taken from each animal Each rabbit was then injected subcutaneously (shallow) with 1 2 I U of insulin, globin insulin or protamine zinc insulin using a microsyringe and marking the site of the injection Following a fixed period of time after the injection a second blood sample was taken and the animal killed by injecting chloroform The skin and part of the underlying tissue was then excised at the site of the injection in a circular form about ¾–1 inch in diameter and frozen immediately with solid carbon dioxide The thyroid was also removed and frozen Blood sugar determinations were made by the method of Hagedorn-Jensen A total of 60 rabbits were used in these experiments The radioactivity counts were made on the intact frozen skin samples using a recording counting rate meter and a Geiger Muller counter with a filter permitting the measurement of gamma rays only Control samples were obtained by injecting excised skin patches with the same amounts of material and following the same technique as used with rabbits

RESULTS Conclusions drawn from the results obtained (given in table 1 and figures 1 and 2) depend on the validity of the assumptions that (1) a modification of the insulin through the formation of an azo derivative and the presence of radioactive iodine in the molecule do not alter its hypoglycemic activity, and (2) that the radioactive iodine remains coupled to the insulin at least until the time when it exerts its hypoglycemic action It has been shown (5) (7) that azo derivatives of insulin exert a hypoglycemic action which is not distinguishable from that produced by regular insulin provided that the number of groups introduced is small and that they do not contain strong acidic or basic groups which shift the isoelectric precipitation zone of the insulin in either the acid or alkali direction The blood sugar curves shown in figure 1 also seem to justify the first assumption since in general they are quite typical of curves obtained in the laboratory for unmodified protamine zinc insulin, globin insulin and insulin preparations However, the 1 hour 20 minute value on the protamine zinc insulin curve and the 10 hour 40 minute point on the globin insulin curve are atypical, both values being somewhat lower than those usually found.

TABLE 1

INSULIN						GLOBIN INSULIN						PROTAMINE ZINC INSULIN					
Elapsed time *hr.*	Elapsed time *min.*	Rabbit no.	mgm. % blood sugar Before injection	mgm. % blood sugar When killed	Radioactivity remaining at site of injection	Elapsed time *hr.*	Elapsed time *min.*	Rabbit no.	mgm. % blood sugar Before injection	mgm. % blood sugar When killed	Radioactivity remaining at site of injection	Elapsed time *hr.*	Elapsed time *min.*	Rabbit no.	mgm. % blood sugar Before injection	mgm. % blood sugar When killed	Radioactivity remaining at site of injection
0	40	1	86	61	0.67	0	40	9A	106	90	1.00	0	40	19	104	97	0.79
0	40	2	102	70	0.54	0	40	10A	102	97	0.86	0	40	20	95	86	0.88
0	40	1A	95	65	0.57	0	40	11A	92	66	0.07	0	40	21A	104	102	0.87
0	40	2A	93	54	0.52	0	40	12A	93	66	0.93	0	40	22A	92	84	1.05
1	20	3	102	56	0.58	1	20	11	101	43	0.25	1	20	21	90	72	0.70
1	20	4	93	52	0.44	1	20	12	110	50	0.60	1	20	22	95	63	0.29
1	20	3A	99	39	0.09	1	20	13A	95	61	0.89	1	20	23A	84	75	0.78
1	20	4A	101	48	0.60	1	20	14A	92	59	0.83	1	20	24A	93	70	0.95
2	40	5	97	47	0.35	2	40	13	95	56	0.65	2	40	23	108	75	0.70
2	40	6	101	86	0.47	2	40	14	110	68	0.60	2	40	24	84	88	0.66
2	40	5A	110	25	0.19	2	40	15A	102	56	0.72	2	40	25A	101	77	0.90
2	40	6A	97	63	0.52	2	40	16A	84	36	0.68	2	40	26A	83	79	0.88
5	20	7	83	54	0.28	5	20	15	90	41	0.57	5	20	25	110	79	0.86
5	20	8	83	83	0.10	5	20	16	110	84	0.17	5	20	26	90	57	0.56
5	20	7A	115	75	0.33	5	20	17A	93	48	0.42	5	20	27A	95	75	0.95
5	20	8A	108	104	0.19	5	20	18A	95	68	0.48	5	20	28A	88	101	0.78
						10	40	17	106	83	0.13	10	40	27	97	77	0.30
						10	40	18	97	93	0.33	10	40	28	101	68	0.63
						10	40	19A	93	29	0.58	10	40	29A	88	90	0.77
						10	40	20A	104	32	0.34	10	40	30A	106	75	0.71
												22	20	29	102	93	0.54
												22	20	30	88	65	0.55
												22	20	31A	90	81	0.61
												22	20	32A	84	74	0.56

Radioactivity of reference samples			Radioactivity of reference samples		Radioactivity of reference samples	
1.06	0.96	0.85	0.93	0.85	0.99	0.94
1.18	0.85		0.87	1.16	0.86	1.01
0.98	1.13		1.14	0.92	1.07	1.07

Some support is afforded the second assumption by the fact that very little radioactivity was found in the thyroid glands. Destruction of the radioactive insulin preparations, if it occurred, had not progressed to such an extent that iodine was made available for the thyroid. The fact that radioactive iodine can be utilized by the thyroid has been shown previously (8).

It has been suggested that a correlation exists between the rate of insulin absorption and the intensity of hypoglycemic action (4). This was confirmed by the experiments presented in this paper inasmuch as rapid decrease in radioactivity is usually associated with low blood sugars. The rate in the decrease of radioactivity at the site of injection was the greatest with insulin and correspondingly the hypoglycemia produced by this preparation was the most intense. Dissipation of radioactivity was the slowest in rabbits injected with protamine zinc insulin. Likewise the hypoglycemia produced by this preparation was the least intense and the slowest in onset. The values for globin insulin were intermediate for both hypoglycemia and rate of decrease of radioactivity. Obviously the duration of hypoglycemia is also correlated with the rate at which radioactivity, that is, insulin, disappears from the site of injection. The duration of hypoglycemia with insulin was the shortest, and with protamine zinc insulin the longest.

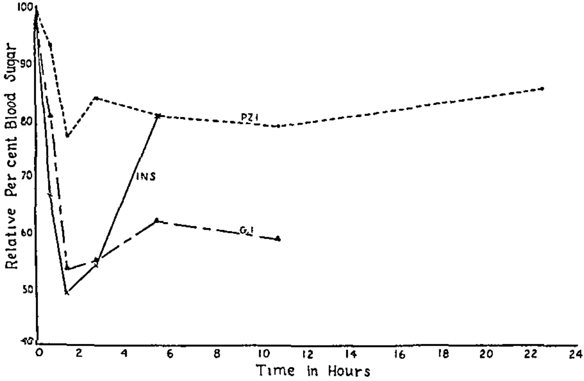

FIG. 1. RELATIVE BLOOD SUGAR CURVES

Ins. = insulin, *G. I.* = globin insulin, and P. Z I. = *protamine zinc insulin; each point* is the average obtained with four rabbits.

The differences in absorption rates of these three preparations are quite marked. For example it requires about 15 times as long for protamine zinc insulin and about 3 times as long for globin insulin as for insulin to reach the level at which 40% of the radioactivity has disappeared. At this point the protamine zinc insulin curve appears to be starting to level off. This occurs about 12 hours following the injection. The globin insulin curve tends to flatten out somewhat about 9 hours after injection at which time about 65% of th radioactivity has disappeared. It is also of interest that insulin (as measure by the radioactivity) disappears from the site of injection about 3 times as fa as does globin insulin until about 60% of the injected materials have disappeared

If diffusion were the process which determines the rate of absorption of insuli one would expect that the logarithm of the residual radioactivity plotted agains time should give a straight line. This was not the case in our experiment The relative rate of absorption of insulin appears to decrease in all cases wit time. This suggests that the process of absorption is rather complex but may also be due to some inhomogeneity in the preparation.

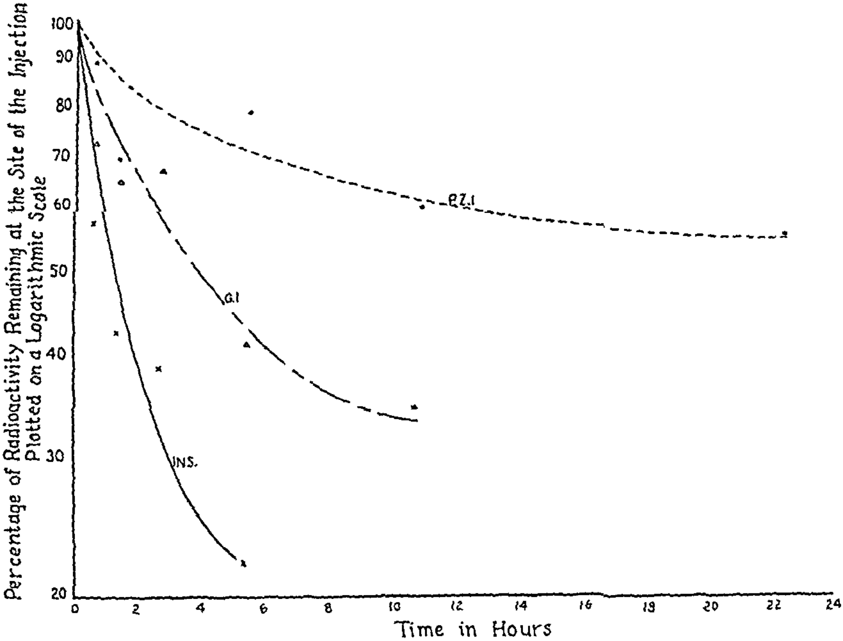

FIG. 2. DISAPPEARANCE CURVES FOR RADIOACTIVE IODINE

Ins. = insulin, G. I. = globin insulin, and P. Z. I. = protamine zinc insulin; each point is the average obtained with four rabbits.

SUMMARY

Radioactive iodine has been used for labelling insulin by coupling diazotized radioactive iodoaniline to insulin. The absorption rate of radioactive insulin injected subcutaneously was compared with its absorption rate from two depot insulin preparations by measuring the decrease of radioactivity at the site of the injection. A correlation was found to exist between the differences in the absorption rate and the differences in the intensity of hypoglycemic action produced by the preparations studied The rate of the absorption of insulin from these preparations was found to be in the following order insulin > globin insulin (with zinc) > protamine zinc insulin

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THE EFFECTS OF ANESTHETIC AGENTS ON MUSCULAR CONTRACTION

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The use of an extract of curare (Intocostrin—Squibb) as a means of improving muscular relaxation during inhalation anesthesia revealed the fact that curare causes more respiratory depression during ether anesthesia than during cyclopropane anesthesia (1). The available evidence seems to indicate that, although there is some direct effect on the respiratory center (2), curare in single therapeutic doses affects peripheral muscular action by interfering with the ability of the muscle cell to respond to the nicotinic action of acetylcholine. It seemed likely, therefore, that the greater respiratory depression which occurs when curare is used during ether anesthesia might be caused by a peripheral action of ether on the humoral mechanism of the transmission of nerve impulses. The investigation was done by determining the response of the gastrocnemius muscle of the dog to intraarterially injected acetylcholine and to electrical stimulation of the sciatic nerve. The agents used were cyclopropane, ether, ethylene, sodium ethyl (*1* methyl-butyl) thiobarbiturate (Sodium Pentothal), and tribromethanol with amylene hydrate (Avertin Fluid).

A curariform action of ether was described in 1914 (3). This action was said to be curariform because no alteration in action currents of the nerve could be demonstrated even when the etherization was of sufficient depth to significantly decrease the magnitude of the muscular response to nerve stimulation by the electric current (4). In cats whose spinal cords were sectioned, the presence of ether in the blood stream decreased the extent of contractions elicited by injecting acetylcholine (5). Pertinent evidence that the interference by ether with the transmission of nerve impulse to muscle cell was humoral was supplied by the observation that eserine potentiated the muscular response to injected acetylcholine in a cat anesthetized with ether (5). Although our results corroborate these observations, other authors (6) could not duplicate them. Their injections of acetylcholine were made, however, into vessels drained of blood and perfused with saline solution. The ether and eserine could not therefore act on the humoral mechanism in the isolated muscle. It is interesting that, in 1914, chloroform was found not to affect the muscular contraction excited by electrical nerve stimuli (7). It is a long established concept that neuromuscular transmission is more susceptible to depression than nerve tissue or striated muscle (8).

METHODS. In order to ascertain whether or not the dog has the same reduced tolerance to curare during inhalation anesthesia with ether that human patients have, four dogs were anesthetized on different occasions with cyclopropane and ether, and 1 mgm. per kgm. of the curare extract was injected intravenously. In the presence of ether, there was a distinct increase in the length of respiratory depression This was characterized by longer periods of complete respiratory arrest and prolongation of the period of intercostal paralysis One dog was also anesthetized in the same fashion and injected with 1 mgm per kgm of *d* tubocurarine This is the active principle in the curare extract and is said not to have any direct effect on the respiratory center (9) This dog however, behaved as did the others that were given the extract of curare These dogs as well as all animals used in this investigation were premedicated with 1 mgm of morphine sulphate per kgm and 0 65 mgm of scopolamine hydrobromide

Muscular responses to injected acetylcholine and electrical nerve stimulation were obtained in the following manner The dog was anesthetized by means of the carbon dioxide absorption technic An endotracheal catheter was introduced and connected to the closed system, and the anesthetic agent and oxygen were added as needed to maintain anesthesia and insure adequate oxygenation at all times The concentrations of the anesthetic agent in the rebreathing bag and blood were not determined The depth of anesthesia was estimated from the animals response The gastrocnemius muscle was then freed and the tendon severed Care was taken not to disturb the blood or nerve supply The tendon was connected to a heavily weighted lever which recorded on a kymograph The lever had a 6 1 ratio of advantage The leg was supported by a clamp at the lower end of the tibia and fibula and braced by a rod placed in the popliteal space The femoral artery was exposed and dissected free until it dipped into the popliteal space One of the small branches closest to this point was cannulated and the cannula connected to a tuberculin syringe The injection of acetylcholine this close to the blood vessels supplying the gastrocnemius muscle produced consistent results The injections were made as rapidly as possible Although the amount of acetylcholine which was injected varied the total volume of fluid was always constant The sciatic nerve was exposed proximally at its entrance into the leg In some experiments it was severed in others it was not

The electrode pierced the nerve and was fixed at this point Stimulation was obtained by the momentary closing of a spring key The current was supplied from a Lovett Garceau stimulator with the frequency set at 120 per second The output of the apparatus could be varied from a minimal stimulus arbitrarily termed 0 on the scale to a maximal stimulus registered as 20

RESULTS Figure 1 is representative of the consistent results obtained in four *dogs in which acetylcholine was injected intra arterially with the sciatic nerve* intact In Section A, the dog was under moderate cyclopropane anesthesia (Stage III, Plane ii), and had been for at least one hour Injection of 0 5 mgm acetylcholine gave a significant contraction The cyclopropane was then washed out of the dog by flushing the closed system with oxygen, and the dog awakened to the point of generalized muscular movement In order to be reasonably certain that the preceding agent was practically eliminated, the system was washed out several times with the second agent used The dog was then anesthetized with ether to the same depth as with cyclopropane The muscular response to 0 5 mgm of acetylcholine, as shown in Section B, failed to occur, and only a minimal contraction was obtained with 1 mgm An attempt was then made to wash out the ether, and the dog was awakened to the point of exhibiting general muscular movement The animal was then reanesthetized with cyclopropane to Plane iii (deep anesthesia) Section C shows that there was a return of ability of the muscle to respond to the acetylcholine The contraction was less than in Section A, but, undoubtedly, a considerable amount of ether was still present in the blood stream and lipoid tissue It is well known that cyclopropane is rapidly eliminated, whereas ether leaves the tissues slowly

In spite of the evidence, already mentioned (4), that ether does not interfere with the conduction of impulses through nerve tissue, it was thought advisable to repeat this experiment with the sciatic nerve sectioned. In experiments on two dogs the difference in the response to cyclopropane and ether remained the same.

One dog was anesthetized with a nonasphyxial concentration of ethylene (80% ethylene, 20% oxygen). As shown in Section A, figure 2, the muscular contraction was satisfactory with 0.1 mgm. of acetylcholine. The ethylene was then discontinued and the dog allowed to inhale 100 per cent oxygen. When

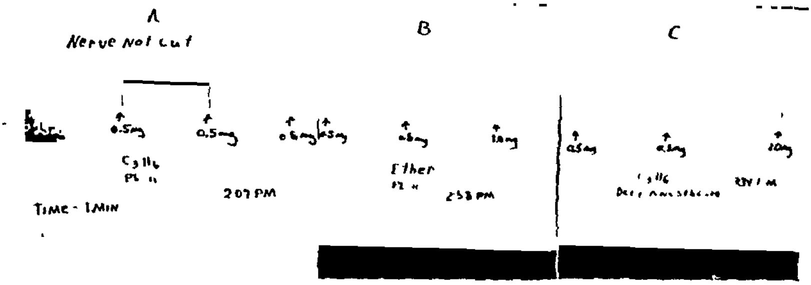

FIG. 1. A = Contraction of gastrocnemeus muscle from the intra-arterial injection of acetylcholine during moderate cyclopropane anesthesia.
B = Same during ether anesthesia of same depth.
C = Same after return to cyclopropane anesthesia of greater depth.

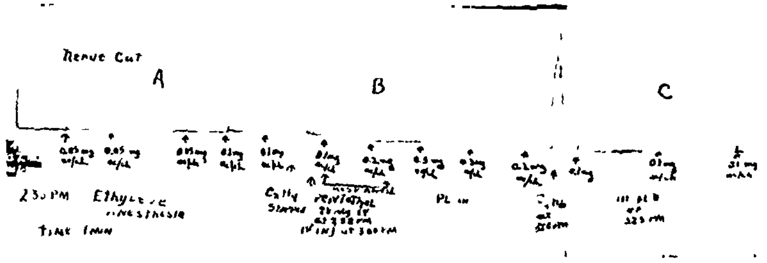

FIG. 2. A = Contraction of gastrocnemius muscle produced by the intra-arterial injection of acetylcholine during ethylene anesthesia.
B = Same under pentothal anesthesia.
C = Same after partial recovery from pentothal and during moderate anesthesia with cyclopropane.

general muscular movement returned, 0.075 gram of sodium pentothal was injected intravenously. This amount produced respiratory arrest which lasted approximately five minutes. During this time, artificial respiration was employed to prevent anoxemia. Even after the resumption of spontaneous respiration considerable more acetylcholine was required (0.2–0.3 mgm.) to effect the same magnitude of contraction that was elicited under ethylene anesthesia (Section B, figure 2). Later, when it was estimated that the blood level of the barbiturate was minimal, this same dog was given cyclopropane, and, as shown in Section C, figure 2, a satisfactory contraction was obtained with only 0 1 mgm of acetylcholine, indicating improved ability of the muscle cell to respond

The production of acetylcholine by the nerve takes place at the terminal filaments within the muscle cell When acetylcholine is injected intra-arterially it might not achieve a comparable effect We thought it advisable, therefore, to attempt a more physiologic excitation of the humoral mechanism by electrical stimulation of the sciatic nerve Figure 3 shows the observations on one dog These observations were consistent in experiments on five dogs It will be seen from Section A, figure 3 that quite satisfactory contractions were obtained with a minimal stimulus with the dog in a moderate plane of cyclopropane anesthesia (Stage III, Plane ii) The cyclopropane was then discontinued and washed out of the dog with oxygen Ether was added after the dog had regained general muscular movement, and the anesthesia was carried to the same plane as it had been with cyclopropane After only five minutes of ether, there was definite diminution in the magnitude of the contraction with the minimal stimulus (Section B, figure 3) After twelve minutes of ether administration, but with no increase in the depth of anesthesia the minimal stimulus failed to elicit a significant contraction (Section C, figure 3) Only when the stimulus was increased to a scale reading of 7 was there a definite contraction (Section E figure 3)

The dog was then given 0 5 mgm of prostigmine methylsulfate intravenously The anesthesia was maintained at the same level Five minutes after the prostigmine had been given, a marked contraction occurred with the Garceau scale reading at 7 (Section F, figure 3) It was now possible to obtain a significant response to the minimal stimulus (Section H, figure 3) An attempt was then made to eliminate the ether, and after the dog showed general muscular activity, cyclopropane was added In the presence of cyclopropane and prostigmine, the magnitude of contraction with the minimal stimulus was greater than that which occurred in the presence of ether (Section I, figure 3) The cyclopropane was then washed out and the dog again given ether, and the contraction with the minimal stimulus was less than that occurring in the presence of cyclopropane (Section J, figure 3)

Figure 4 shows a comparison in one dog of the muscular contraction during deep anesthesia with cyclopropane (Stage III, Plane iii) and deep anesthesia with tribromethanol with amylene hydrate (Avertin Fluid) Section A shows the magnitude of the contraction with a minimal stimulus during anesthesia with cyclopropane The dog was then given 200 mgm per kgm of a 3% solution of Avertin Fluid intraperitoneally, and the cyclopropane eliminated by flushing the system with 100% oxygen As can be seen in Section C, figure 4, there was only moderate reduction of the magnitude of the contraction with a minimal stimulus even in anesthesia deep enough to cause respiratory arrest During this period artificial respiration with 100% oxygen was performed

With the return of spontaneous respiration and a lighter plane of anesthesia (Stage III Plane ii) the magnitude of the response to a minimal stimulus was not less than that during anesthesia with cyclopropane (Section E figure 4)

Figure 5 illustrates the potentiation of the response to minimal stimuli caused

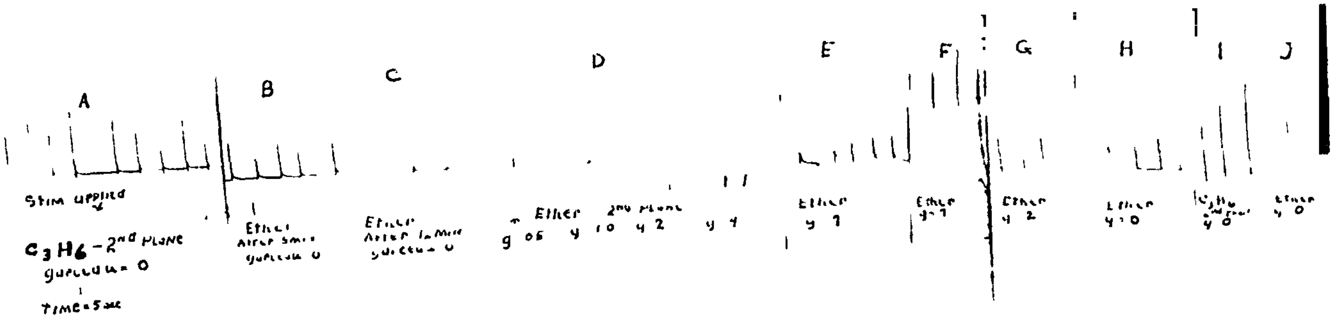

FIG. 3. *A* = Contraction of gastrocnemius muscle with stimulation of sciatic nerve during moderate cyclopropane anesthesia. Stimulator reading = 0.

B = Contraction with same intensity of stimulation after 5 minutes ether administration.

C = Same as B after 12 minutes of ether administration.

D and *E* = Contraction with stimulation of sciatic nerve during moderate ether anesthesia. Stimulator reading = 4 and 7.

F = Contraction with nerve stimulation during moderate ether anesthesia, 5 minutes after 0.5 mgm. of prostigmine I.V. Intensity of stimulus same as in E.

G and *H* = Same as F except intensity of stimulus reduced. Stimulator reading = 2 and 0.

I = Dog allowed to partially recover from ether anesthesia and then anesthetized to moderate depth with cyclopropane. Stimulator reading = 0.

J = Reanesthetized to same depth with ether. Stimulator reading = 0.

by the administration of prostigmine methylsulfate during cyclopropane anesthesia in three dogs This potentiation persists when the agent is changed to ether.

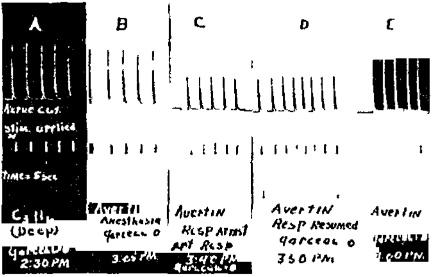

Fig [illegible] imal stimulation of sciatic nerve d [illegible] Stimulator reading = 0
B = [illegible]
C = [illegible] piratory arrest, (artificial respiration)
D = Same during tribromethanol anesthesia after respiration resumed
E = Stimulation during tribromethanol anesthesia 10 minutes after respiration resumed

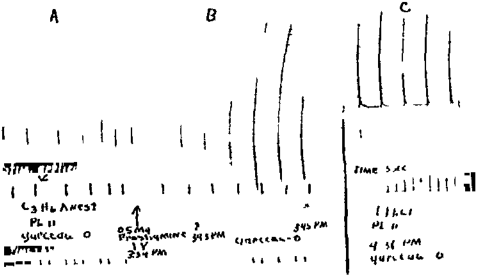

Fig 5 *A* = Contractio [illegible] sciatic nerve during moderate cyclopropane anesthesia
B = Same after 0 5 mgr [illegible]
C = Contraction with s [illegible] sciatic nerve during moderate ether anesthesia

DISCUSSION These observations show that the anesthetic agents which were studied affect the humoral transmission of nerve impulses, i e , the ability of the muscle cell to respond to acetylcholine injection and to electrical stimulation of the nerve. It seems unlikely that the difference in response is due to changes in the ability of the nerve to conduct impulses. The results were consistent, regardless of whether the nerve was intact or severed. As stated earlier, ether was found not to reduce the electrical disturbance in the nerve (4). In addition, it can be inferred from the observations of Brown *et al.* (6), that the agent must come in contact with the nerve endings and muscle cell to alter the contraction response. Since our investigation of this problem was stimulated by the respiratory depression which occurs with the administration of curare during ether anesthesia, it is of interest, also, that observations were made in 1914 (10) which indicated that "probably the intoxication of the peripheral respiratory mechanism has some share in the early stoppage of the respiration by ether anesthesia." Our experience with *d*-tubocurarine corroborates the significant peripheral effect.

The exact site of interference is not clear. The agent could depress or stimulate the activity of cholinesterase or depress or stimulate the receptor substance in the muscle cell. The potentiation of the contraction by prostigmine gives further evidence that the interference with contraction is located in the humoral mechanism, but does not necessarily indicate a previous depression of acetylcholine or stimulation of cholinesterase by the anesthetic agent. There still might be depression of the receptor substance, which was capable, however, of responding to the acetylcholine when its hydrolysis was delayed by depression of the cholinesterase with prostigmine.

There is a report of *in vitro* experiments which indicates that the gaseous and volatile anesthetic drugs and the nonvolatile tribromethanol and evipal do not inhibit the activity of cholinesterase (11). It has also been stated from observations made on rats that the production of acetylcholine is increased with cyclopropane and diminished with ether and ethylene (12). Another report adduces evidence from *in vitro* experiments that cholinesterase is inhibited by ether and chloroform (13). The latter author suggests that the actions of prostigmine and ether and chloroform differ only quantitatively. If this were the only effect *in vivo* we would not have observed the progressive depression of muscular contraction with ether as shown in figure 3 but rather a progressive enhancement of that contraction. Additional contradictory evidence of depression of cholinesterase by chloroform is the lack of increased magnitude of muscle contraction during chloroform anesthesia *in vivo* (7). The depression of muscular contraction during ether anesthesia probably is due to inhibition of acetylcholine or of receptor substance. It is well established that curare acts on the receptor substance in striated muscle. In our experiments and in clinical application, ether seems to potentiate this action. Although we are unable to differentiate the site of action of ether from our experimental evidence, it seems most probable that ether acts to depress the receptor substance.

SUMMARY

From the observations recorded in this investigation, it can only be said that (*1*) the contraction of the gastrocnemius muscle which is elicited by intra-arterially injected acetylcholine or electrical stimulation of the nerve is less

pronounced in dogs anesthetized with ether, tribromethanol and sodium pentothal than in dogs anesthetized with cyclopropane or ethylene (2) prostigmine potentiates the contraction response in the dog anesthetized with cyclopropane and ether and (3) the difference in contraction during anesthesia with the various agents is not altered by sectioning the nerve supply to the muscle

It seems likely therefore, that the interference with the contraction is located in the humoral mechanism of transmission of nerve impulses and that ether, tribromethanol and sodium ethyl (1 methyl butyl) thiobarbiturate (Sodium Pentothal) can be said to have a curariform action Of these three ether causes the greatest interference tribromethanol and sodium pentothal do so only in very high blood concentrations

The clinical difficulties encountered with the concomitant use of ether and curare are thus accounted for

Acknowledgement The Intocostrin used in these experiments was supplied by courtesy of E R Squibb & Sons New York

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THE FATE OF SULFONAMIDES AND *p*-AMINOBENZOIC ACID IN COLD-BLOODED ANIMALS

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The wide use of the chemical method of determining sulfonamides in both blood and urine, as devised by Marshall, Emerson, and Cutting (1), has resulted in rational use of these chemotherapeutic agents. By the same procedure, Marshall and his associates (2, 3) discovered that all the derivatives of sulfanilamide so far examined undergo partial acetylation in rabbits and men while they are excreted unchanged in dogs and frogs. Conjugation (presumably acetylation) also occurs in fish, chickens, mice, rats, guinea pigs, cats, cows, pigs, horses, and monkeys (4). In the interest of comparative pharmacology, the following series of experiments were carried out to ascertain the fate of sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine in other cold-blooded animals than frogs and fish. Although *p*-aminobenzoic acid has an antagonistic action toward sulfonamides, its elimination in the body is closely similar to that of sulfonamides, namely, it is acetylated in rabbits and men but unaltered in dogs (6, 7, 8). It is for this reason that it was included in the investigation.

Procedures. The animals employed were the spadefoot toad, *Scaphiopus holbrookii holbrookii*, the nebulous toad, *Bufo valliceps*, the turtle, *Pseudemys elegans*, the Leopard frog, *Rana pipiens*, the "Jumbo" bullfrog, *Rana catesbeiana*, and the tree frog, *Hyla cinerea cinerea*. A 1% solution of the sodium salt of sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, and *p*-aminobenzoic acid was freshly prepared. The testing dose in all animals was 0.2 mgm. per gram. Following injection of the drug into the ventral lymph sac, the spadefoot toad, the nebulous toad, the Leopard frog, and the tree frog were placed in individual, perforated cages immersed in a water bath, 1.8 cm. deep, the temperature of which varied from 23° to 33°C. depending on the room temperature. They were sacrificed at various intervals by pithing. After the removal of the chest and abdominal walls, blood samples were taken by ventricular puncture, and urine samples by aspiration from the urinary bladder. Obviously each animal could be bled and aspirated only once. The amount of the administered drug in both blood and urine of each animal was determined in a photoelectric colorimeter (photelometer) by the revised method of Bratton and Marshall (9), a volume of 0.1 cc. of each specimen being used, making a final dilution of 1:100. In every instance an attempt was made to determine the conjugated as well as the free form.

Regarding the turtle, the test drug was injected intramuscularly after pithing and the removal of the plastron. The turtle was laid on its back and covered with a wet towel during experimentation. Blood samples were drawn from the ventricle every ½ hour for 2 hours following medication, and at hourly intervals thereafter, for a total period of 7 hours. This was possible because the turtle has a relatively larger blood volume than the above 4 smaller animals. For the same reason, the "Jumbo" bullfrog could be bled repeatedly at definite intervals following medication by the lymph sac and the destruction of the central nervous system. Urine specimens were also obtained.

Results. Since all the small animals, namely, the spadefoot toad, the nebulous toad, the Leopard frog, and the tree frog, each contributed only one reading to the concentration of the sulfonamides and *p* aminobenzoic acid in blood and urine it was necessary to employ a considerable number of animals, sacrificed at various intervals, in order to ascertain the general course of absorption and elimination In table 1 it will be seen that in a group of 25 spadefoot toads the average blood concentration of free (non conjugated) sulfanilamide

TABLE 1

The spadefoot toad

DRUG	NUMBER OF ANIMALS USED	AVERAGE* CONCENTRATION (MCM PER 100 CC)										
		Material examined	Form determined	Time after administration (hours) ½	1	1½	2	3	4	5	6	7
Sulfanilamide	25	Blood	Free	17 1	17 1	15 6	15 2	10 0	13 4	12 0	10 6	12 6
			Conjugated	0 3	0 3	0 0	0 0	0 5	0 5	0 2	0 2	0 0
		Urine	Free	8 6	14 7	12 7	16 8	11 6	14 1	16 8	14 1	15 3
			Conjugated	0 0	0 0	0 0	0 1	0 3	0 5	0 8	0 7	0 0
Sulfapyridine	28	Blood	Free	23 9	21 0	20 5	18 7	16 8	16 8	13 4	9 9	10 6
			Conjugated	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 5
		Urine	Free	20 6	22 8	26 9	†	22 2	20 5	20 7	26 8	17 6
			Conjugated	0 0	1 0	0 0	†	2 3	2 1	1 3	2 1	4 7
Sulfathiazole	28	Blood	Free	28 0	23 2	25 1	17 4	18 1	17 2	16 5	11 5	12 0
			Conjugated	0 0	0 0	0 0	0 1	0 2	0 4	0 3	1 4	0 3
		Urine	Free	16 9	37 3	34 3	30 2	30 2	27 7	20 0	28 0	28 6
			Conjugated	0 0	1 2	0 3	3 4	2 6	5 8	4 0	0 0	10 3
Sulfadiazine	25	Blood	Free	23 8	24 3	24 5	16 5	21 2	18 3	18 3	14 7	14 8
			Conjugated	0 0	0 0	0 0	0 0	0 0	0 3	0 0	0 4	0 0
		Urine	Free	16 3	22 0	35 4	32 9	30 9	46 4	40 2	32 8	45 6
			Conjugated	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 4	0 7
p Aminobenzoic acid	25	Blood	Free	18 4	17 0	†	14 7	12 2	12 6	10 7	7 6	5 1
			Conjugated	0 0	0 1	†	0 8	0 7	1 3	1 4	2 1	1 7
		Urine	Free	24 6	19 7	†	25 7	28 0	18 5	31 6	12 4	16 3
			Conjugated	0 0	0 0	†	0 6	6 0	1 7	5 2	12 6	20 0

* Average of 2 to 4 animals

† Not determined

calculated as such reached its maximum at the end of ½ hour after injection The average urine concentration attained its highest level at the end of the second hour, and remained high thereafter The conjugated form of sulfanilamide (total minus free) was present but was small in amount in both blood and urine When compared with that of the rabbit the degree of conjugation in the spadefoot toad s blood is much less significant, as shown in Figure 1 Sulfa pyridine, sulfathiazole, sulfadiazine, and *p*-aminobenzoic acid were also promptly absorbed—the blood concentration of the free form being highest ½ hour after injection for three of the compounds and 1½ hours after injection for sulfadiazine. There was a slight conjugation, most noticeable with *p*-aminobenzoic acid. The excretion in urine increased progressively, reaching the maximum in from 1½ to 7 hours. The conjugated form, except that of sulfadiazine which behaved like sulfanilamide, was relatively high in urine as compared with that in blood. It is probable that the free form of these substances is more easily reabsorbed through the kidney tubules than the conjugated form.

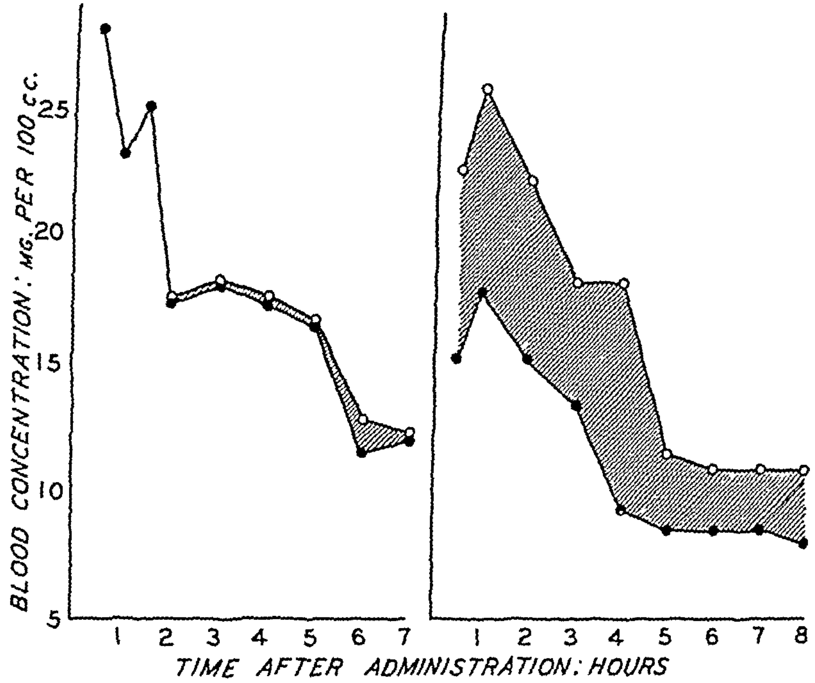

FIG. 1. COMPARISON OF CONJUGATION OF SULFATHIAZOLE IN THE SPADEFOOT TOAD AND THE RABBIT

The graph on the left-hand side represents the average blood concentration of sulfathiazole in 28 spadefoot toads each injected by the ventral lymph sac with 0.2 mgm. of sodium sulfathiazole per gm. of body weight. The curve through the solid circles designates the free form, and that through the hollow circles the total concentration. The shaded area indicates the amount of conjugation.

The graph on the right-hand side is the blood concentration of a rabbit, female, weighing 2.32 [illegible] of sodium sulfathiazole in the dose of 200 mgm. per [illegible] ween the two animals is very evident.

In general, the nebulous toad has a trend of response similar to that of the spadefoot toad. As shown in table 2, the conjugated form in the blood was relatively low in each case. With sulfadiazine the conjugated form was not detectable at all in the blood, although it could be definitely determined in the urine The appearance of the conjugated form in urine and not in blood makes a difficult case for explanation It must be assumed that concentration takes place in the kidney, so that its level, originally imperceptible, becomes finally measurable Certainly it is less probable that conjugation occurs in the kidney

TABLE 2

The nebulous toad

DRUG	NUMBER OF ANIMALS USED	AVERAGE* CONCENTRATION (MGM PER 100 CC)											
		Material examined	Form determined	Time after administration (hours)									
				½	1	1½	2	3	4	5	6	7	8
Sulfanilamide	24	Blood	Free	14 5	15 4	17 0	13 1	12 3	12 3	9 7	8 3	8 1	7 0
			Conjugated	0 0	0 7	1 6	1 2	1 3	2 4	1 9	2 8	1 6	1 6
		Urine	Free	16 4	12 0	†	9 7	16 7	15 8	12 3	9 9	10 5	9 0
			Conjugated	1 2	0 6	†	2 4	3 8	6 6	10 1	16 7	4 3	10 6
Sulfapyridine	25	Blood	Free	12 8	11 6	9 7	9 4	8 2	8 3	5 4	4 0	4 4	5 5
			Conjugated	0 0	0 5	1 5	0 8	1 7	1 4	1 4	1 3	1 1	0 0
		Urine	Free	10 0	12 8	12 4	14 5	13 6	9 2	11 2	13 0	12 8	8 8
			Conjugated	2 0	3 9	6 5	7 8	7 4	9 2	9 9	12 2	10 8	15 0
Sulfathiazole	25	Blood	Free	16 3	16 0	16 9	14 8	14 1	14 9	12 5	10 5	8 9	†
			Conjugated	0 0	0 5	0 0	0 2	0 2	0 2	0 0	0 0	0 6	†
		Urine	Free	†	21 2	21 4	22 5	35 8	23 2	28 3	46 0	18 4	†
			Conjugated	†	0 8	1 2	2 5	11 2	7 0	11 0	12 0	7 6	†
Sulfadiazine	29	Blood	Free	19 0	21 8	22 5	18 2	16 1	13 2	13 5	10 2	8 8	9 4
			Conjugated	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
		Urine	Free	14 4	26 4	30 7	34 0	36 9	31 3	32 9	32 4	38 0	23 4
			Conjugated	0 0	0 0	0 0	0 0	3 1	3 7	3 8	4 1	7 2	3 5
p Aminobenzoic acid	28	Blood	Free	18 1	17 5	17 0	14 8	10 4	8 8	7 4	4 3	2 5	†
			Conjugated	0 0	0 7	0 6	0 9	2 3	3 3	3 1	2 3	3 8	†
		Urine	Free	17 5	20 9	21 4	25 9	21 7	11 1	3 4	4 4	8 4	†
			Conjugated	4 1	4 0	5 4	16 9	35 3	22 9	40 4	48 6	50 6	†

* Average of 2 to 4 animals

† Not determined

The excretion of the conjugated form of sulfanilamide sulfapyridine, sulfathiazole and *p* aminobenzoic acid in urine mounted progressively

In confirmation of Marshall s work (3) the Leopard frog was found to have no conjugated form in either blood or urine with the 4 sulfonamides or *p* amino benzoic acid Similarly the Jumbo bullfrog did not show any conjugated form of sulfanilamide sulfapyridine or sulfathiazole On the other hand in the tree frog conjugation was definitely observed in both blood and urine with sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine.

As stated above, the blood of the turtle could be repeatedly examined for the concentration of the drugs injected. From the results presented in table 3, it can be stated that no conjugation takes place with sulfanilamide, sulfapyridine, sulfadiazine, or *p*-aminobenzoic acid, but it was demonstrable, though slightly, with sulfathiazole. Incidentally, the concentration of the free form in each case remained at a high level throughout the period of 6 to 7 hours. This might be due to dehydration since the urinary bladder was collapsed in 6 out of 8 animals. It might also be due to the progressive weakening of the heart beat following the first puncture. Another peculiar feature was that on 4 occasions when the urine was examined, neither the free nor the conjugated form of the administered drug was detectable. It was possible, however, that no urine was actually excreted following the injection, owing to an inadequate filtration pressure as a result of the feeble heart beat.

TABLE 3

The turtle

DRUG	NUMBER OF ANIMALS USED	AVERAGE CONCENTRATION (MGM. PER 100 CC.)										
		Material examined	Form determined	Time after administration (hours)								
				½	1	1½	2	3	4	5	6	7
Sulfanilamide	2	Blood	Free	3.3	3.1	3.4	3.5	4.9	7.4	7.4	8.2	9.2
			Conjugated	0.0	0.0	0.0	0.0	0.0	0.0	0 0	0.0	0.0
Sulfapyridine	3	Blood	Free	14.2	13.5	12.9	12.5	12.9	13.1	11.9	12.3	8.0
			Conjugated	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sulfathiazole	3	Blood	Free	14.5	24.7	20.2	26.5	16.2	15.2	21.6	13.5	14.6
			Conjugated	0.4	0.6	0.2	0.0	0.2	0.1	0.4	0.1	0.0
		Urine	Free	†	†	†	0.0	†	†	†	0.0	†
			Conjugated	†	†	†	0.0	†	†	†	0.0	†
Sulfadiazine	2	Blood	Free	19.6	16.7	16.2	15.8	14.9	15.2	15.1	18 0	†
			Conjugated	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	†
		Urine	Free	†	†	†	0.0	†	†	0.0	†	†
			Conjugated	†	†	†	0.0	†	†	0.0	†	†
p-Aminobenzoic acid	2	Blood	Free	29.0	34.6	35.5	34.5	30.6	26.1	23.8	26.5	†
			Conjugated	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	†

† Not determined.

SUMMARY

1. Sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, or *p*-aminobenzoic acid, in the form of the sodium salt, when injected into the ventral lymph sac

of the spadefoot toad (*Scaphiopus holbrookii holbrookii*), or the nebulous toad (*Bufo valliceps*), is partly conjugated as shown in blood and urine The degree of conjugation as observed in blood is apparently not as great as in the rabbit In the nebulous toad's blood, the conjugated form of sulfadiazine is unmeasurable

2 Similarly, conjugation takes place in the tree frog (*Hyla cinerea cinerea*) with sulfanilamide, sulfapyridine, sulfathiazole, and sulfadiazine

3 Confirmatory of Marshall's work, sulfanilamide, sulfapyridine, and sulfathiazole are found to be unconjugated in the Leopard frog (*Rana pipiens*) and the "Jumbo" bullfrog (*Rana catesbeiana*) Also, sulfadiazine and *p* aminobenzoic acid are not conjugated in the former animal

4 No conjugation takes place in the turtle (*Pseudemys elegans*) with sulfanilamide, sulfapyridine, sulfadiazine, or *p* aminobenzoic acid There is, however, a slight conjugation with sulfathiazole

Acknowledgment We are indebted to Messrs Frank A Steldt and Harold Worth for their invaluable assistance

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THE ACTION OF RIDDELLINE

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Riddelline, $C_{18}H_{23}O_6N$, is an alkaloid of *Senecio riddellii* and was first isolated by Manske (1). By alkaline hydrolysis, Adams, Hamlin, Jelinek, and Phillips (2) obtained retronecine and riddellic acid. Chemically, therefore, riddelline resembles other *Senecio* alkaloids. With the exception of platyphylline, the majority of the *Senecio* alkaloids so far investigated produce well defined lesions of the liver in experimental animals (3, 4, 5, 6, 7, 8). In order to ascertain whether or not riddelline possesses a similar action, the following series of experiments were carried out.

Our material was courteously supplied by Dr. Richard H. F. Manske, Division of Chemistry, National Research Council, Ottawa, Canada, and Professor Roger Adams, Department of Chemistry, University of Illinois, Urbana. Fresh solutions of riddelline, 1% being most suitable, were prepared by dissolving the weighed quantity in an equimolecular amount of hydrochloric acid. Albino mice were injected intravenously with various doses of riddelline, and the survivors observed for a week. A total of 119 animals was used.

The results are summarized in table 1. Several mice receiving doses of 125–160 mgm. per kgm. developed clonic convulsions, but all recovered temporarily. They and others injected with smaller doses gradually became motionless with hair standing on end, lost their appetite for food, and died within 24–96 hours. Based on 1 week's observation in 35 mice, the median lethal dose (LD_{50}) of Dr. Manske's specimen was found to be 108.8 ± 3.06 mgm. per kgm. as reported previously (9); that of Professor Adams' specimen, in 84 mice, 97.74 ± 5.60 mgm. per kgm. Since the difference is not statistically significant, the data are combined and the LD_{50} is recomputed to be 104.9 ± 4.15 mgm. per kgm. as shown in table 1—a case of biological confirmation of the chemical identity of two products.

Of 40 mice examined at necropsy following spontaneous death, 20 had ascites, 14 had hydrothorax, and 5 had pulmonary edema. The thymus glands of 12 of these animals were examined microscopically and necrosis of cortical lymphocytes was found in 7. As was the case with the administration of other *Senecio* alkaloids, the liver was uniformly affected. Necrosis took place, extensive or moderate in 33, and slight in 7. Thirty-seven livers showed central necrosis; 2, predominantly midzonal; and 1, periportal. Figure 1 may be taken as a typical example of central liver necrosis. Frequently the cells adjacent to the necrotic zone contained much fat. As was true with other *Senecio* alkaloids, there was usually no or only slight leukocytic invasion of the necrotic tissue. In association with necrosis, sinusoidal congestion with hemorrhage into the cords of necrotic cells frequently occurred—extensive or moderate in 34, and

TABLE 1

Toxicity of riddelline

DOSE	NUMBER USED/NUMBER DIED	LD50 ± S.E.	NUMBER AUTOPSIED	
			After death	After sacrifice
mgm./kg.		*mgm./kg.*		
50	0/5			4
62	0/10			5
70	1/10		1	4
80	4/15		4	7
90	6/25	104.9 ±4.15	6	10
100	8/20		7	12
110	6/14		6	8
125	9/10		7	1
140	4/5		4	1
160	5/5		5	

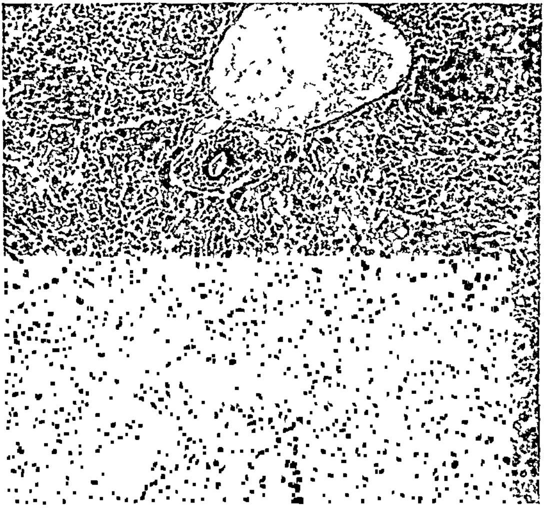

FIG. 1. CENTRAL NECROSIS OF LIVER PRODUCED BY RIDDELLINE. ×174

Mouse number 93, female, weighing 15.8 gm., received an intravenous dose of 110 mgm. per kgm. of riddelline. It died 3 days later. There is extensive central necrosis with extension in places to the margins of the portal spaces. Many small darkly stained masses of chromatin are visible. These are derived mainly from necrotic liver cells although some may have come from the nuclei of leukocytes.

slight in 5. In some instances hemorrhage was so severe that many liver cells seemed to have been washed away—a picture first noted by Davidson (4) in rats following administration of retrorsine. Occasionally the congestion and hemorrhage were more or less circumscribed, producing an appearance we have previously referred to as angiomatoid and have illustrated elsewhere (7, fig. 1).

Of the 61 mice which were chloroformed 7 days after administration of the drug, the livers of 4 showed one or two grossly visible foci of necrosis. Tissues of 40 were examined microscopically, and all but 7 appeared normal. The liver of 1 mouse which received 80 mgm. per kgm. showed slight hydrops, a lesion probably not due to the alkaloid. The livers of 4 mice which received 90 mgm. per kgm. contained foci of necrosis involving one or more entire lobules. In the livers of these animals and of 2 others, some parenchymal cells were missing from the centers of some lobules and in their place were monocytes.

In addition to the toxicological study, a few experiments were conducted to test the action of riddelline on smooth muscle organs. The alkaloid inhibited the isolated small intestine of the rabbit—moderately in a 1:40,000 concentration, and definitely in a 1:20,000 concentration. It stimulated the isolated guinea pig's uterus in a 1:10,000 solution. In pithed or barbitalized cats, doses of 20, 50, and 120 mgm., injected intravenously, lowered the arterial blood pressure with recovery. Only in one cat, anesthetized by ether, did a dose of 20 mgm. cause a slight rise of blood pressure.

SUMMARY

1. The acute toxicity of riddelline has been determined in mice by intravenous injection.

2. Like other *Senecio* alkaloids so far studied, riddelline produces in mice central necrosis of the liver, ascites or hydrothorax, and pulmonary edema. There is also evidence of necrosis of cortical lymphocytes in the thymus.

3. Riddelline inhibits the isolated rabbit's small intestine and stimulates the isolated guinea pig's uterus. It causes a fall of blood pressure when injected by vein in pithed or barbitalized cats.

Acknowledgment. We are indebted to Messrs. Frank A. Steldt and Harold Worth for their invaluable assistance.

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THE INACTIVATION OF CHOLINESTERASE BY MORPHINE, DILAUDID, CODEINE AND DESOMORPHINE[1]

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Bernheim and Bernheim (1) found that morphine inhibited the action of brain cholinesterase They used the ileum of the guinea pig as a test object for the presence of acetylcholine Kuhn and Searles (2), using the same method, reported similar results Slaughter and Lackey (3), using a titrametric method, were unable to find any *in vitro* inhibition of the cholinesterase in dog serum

In the meantime Slaughter and Gross (4) reported that eserine potentiated the *in vivo* action of morphine on the intestine and blood pressure, and Slaughter and Munsell (5) reported that prostigmin potentiated the analgesic effect of morphine as measured by the pain response of the cat Since these effects could be explained by an inhibition of cholinesterase, a detailed study of the action of morphine and some of its derivatives on the *in vitro* activity of cholinesterase was undertaken During the progress of this work Eadie (6) published the results of a comprehensive study of the action of morphine on the cholinesterase of dog serum

METHOD The rate of hydrolysis of acetylcholine was measured by the method of Ammon (7) This method depends on the reaction of the liberated acetic acid with the bicarbonate of the solution, resulting in an evolution of CO_2 which was measured manometrically in Barcroft differential manometers The acetylcholine iodide was dissolved in Ringer bicarbonate solution (8) and 0 5 cc placed in a side arm of the reaction flask The serum or brain suspension and drug were placed in the main compartment of the flask and Ringer bicarbonate added to a total volume of 3 0 cc , including the contents of the side arm The manometers were then placed in a water bath at 37 5°C and flushed for 10 minutes with a mixture of 95% N_2 and 5% CO_2 previous to closing the stopcocks After a period of 5 minutes to assure equilibrium, the contents of the side arm were tipped into the main vessel and readings taken at 10 minute intervals

Venous blood was collected from the rabbit dog or man, and after clotting was placed in the refrigerator to allow clot retraction The serum was decanted after 24 hours and stored in the icebox at 3°C Sections of two human brains[2] were obtained at autopsy four hours *post mortem* and immediately frozen The rabbit brains were removed immediately after the death of the animals The brain cortex only was used The frozen slices were weighed, thoroughly ground with clean sand and Ringer bicarbonate added at intervals during the grinding until the suspension contained 10 mgm (rabbit) or 100 mgm (human) per cc The suspension was stirred well before withdrawing the sample Fresh suspensions were made for use each day

A preliminary examination of each sample of serum or brain was made to determine the cholinesterase activity Quantities of the material were then chosen so that approximately

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[1] A preliminary report of some of this work appeared in the J Pharmacol and Exp Therap 72 45 1941

[2] Obtained through the kind cooperation of Dr F Eberson, Gallinger Hospital, Washington D C

80 mm.[3] of CO_2 were liberated in 45 minutes in the presence of 2.5 mgm. of acetylcholine iodide. From the data obtained it was found that 0.025 cc. of human serum, 0.04 cc. of dog serum, 0.16 cc. of rabbit serum, 8.0 mgm. of rabbit brain, and 80 mgm. of human brain were approximately equivalent in cholinesterase activity in the presence of 2.5 mgm. of substrate. These quantities were used in the experiments reported here.

The morphine, codeine, dihydromorphinone (dilaudid), and dihydrodesoxymorphine-D (desomorphine) were kindly furnished by Dr. L. F. Small of the National Institute of Health. The first three were in the form of hydrochloride salts and the last was a sulphate.

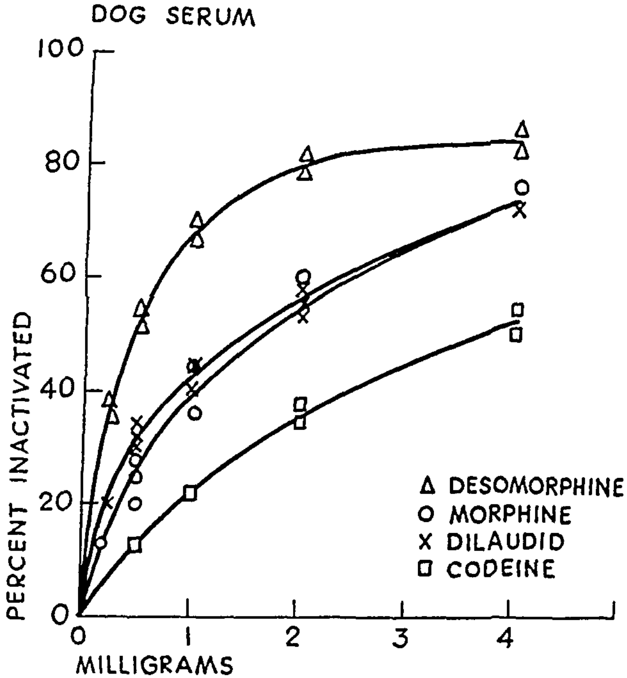

FIG. 1. PER CENT INACTIVATION OF THE CHOLINESTERASE OF DOG SERUM BY THE ALKALOIDS INDICATED

In figures 1 through 5 the abscissae represent the concentrations of the drugs as milligrams in 3.0 cc.

RESULTS. The results obtained with varying concentrations of the inhibitor and a fixed enzyme and substrate concentration are shown in figures 1 to 5. The effect of morphine and three of its derivatives on the activity of dog serum cholinesterase is shown in figure 1. Each of the alkaloids inactivated a portion of the enzymic activity, and the inactivation increased with increasing concentration of the alkaloids. Desomorphine was by far the most effective inhibitor, morphine and dilaudid about equally effective, and codeine the least.

The results obtained with rabbit serum are shown in figure 2. The order of effectiveness was the same as that found using dog serum but the amount of cholinesterase inactivated at a given concentration of the alkaloids was somewhat less for morphine, dilaudid and codeine. A more striking difference in the relative inhibitory effect of the alkaloids was found when human serum was used (figure 3). The order of decreasing effectiveness changed to desomorphine, codeine, morphine and dilaudid. Codeine inactivated more of the enzyme than morphine or dilaudid. Furthermore, a distinct difference was found between morphine and dilaudid, the latter being the least effective inhibitor.

When human brain was used as a source of enzyme (figure 4), the order of effectiveness was the same as that for dog or rabbit serum. In contrast to the results observed with human serum, codeine was the least effective inhibitor. However, dilaudid was a definitely less effective inhibitor than morphine, as was also found when human serum was used.

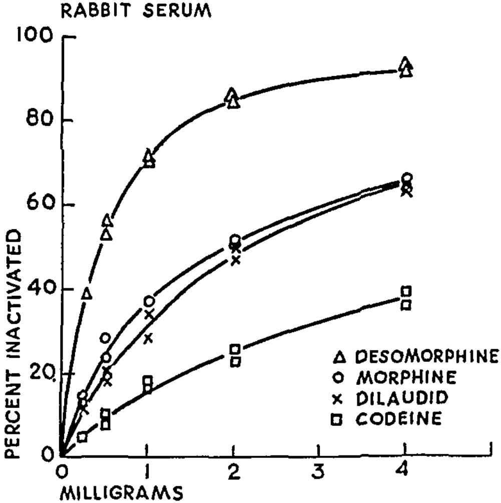

FIG 2. PER CENT INACTIVATION OF THE CHOLINESTERASE OF RABBIT SERUM BY THE ALKALOIDS INDICATED

Figure 5 shows the results obtained when rabbit brain was used as a source of enzyme In this case desomorphine caused a complete inactivation at a concentration of 4 mgm. in 3.0 cc. In other respects the results are not greatly different from those found for human brain.

The results obtained with varying concentrations of substrate and fixed concentrations of enzyme and inhibitor are given in tables 1 and 2. In both tables experimental points as shown in figure 6, we made estimates of the maximum velocities and inflection points graphically, and using these estimates as parameters in equation (*A*) determined the "best" values by the method of least squares, using a series of approximations. This operation was suggested by Dr. Harold Dorn of the National Institute of Health.[3]

TABLE 1

Initial rates of hydrolysis by human serum (.025 cc.) in the presence of constant amounts of inhibitors and varying substrate concentrations

SUBSTRATE MOLAR	CONTROL SERUM	DILAUDID*	MORPHINE*	CODEINE*	DESOMORPHINE*	SPONTANEOUS HYDROLYSIS†
.0061	0.92					
.00122	1.31	0.50	0.31	0.14	0.12	
.00244	1.74	0.72	0.43	0.26	0.12	
.00488	1.99	1.05	0.67	0.50	0.25	0.08
.00976	2.21	1.40	0.93	0.73	0.49	0.16
.0195	2.50	1.68	1.26	0.99	0.74	0.30
.0390	2.61	1.94	1.44	1.23	0.96	0.46
.0781	2.57	2.00	1.67	1.41	1.19	0.77
.110						1.08
.156	2.59	2.10	1.72		1.44	1.25

* The amount of inhibitor used was 4.0 mgm. in 3.0 cc. reaction volume.

† Experimental data. A curve was drawn through these points, and the values for ubstrate concentrations below .00488M were extrapolated.

TABLE 2

nitial rates of hydrolysis by dog serum (.04 cc.) in the presence of constant amounts of inhibitor and varying substrate concentrations

SUBSTRATE MOLAR	CONTROL SERUM	MORPHINE
.156	2.01	1.29
.078	1.98	1.30
.0390	1.98	1.17
.0195	2.02	.99
.00976	1.99	.82
.00488	1.90	.55
.00244	1.76	.46
.00122	1.63	
.00061	1.20	
.00030	.75	
.00015	.60	

It is obvious from the four lower curves that in the presence of the indicated inhibitors K_s is definitely increased and V decreased. This indicates that both competitive and non-competitive inhibition (10) take place in the presence of these inhibitors. The value for V was 2.61; for V' in the presence of dilaudid,

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[3] See appendix.

2 15, of morphine, 1 77, of codeine, 1 61, and of desomorphine, 1 51 The value for K_s was 0012, which is in close agreement with the value 0011 obtained by Glick (9) Values for K'_s in the presence of the drugs were respectively, 0050, 0069, 012, and 029

K_s can be considered as the dissociation constant of the enzyme substrate complex, and its reciprocal the relative affinity of enzyme for substrate In the

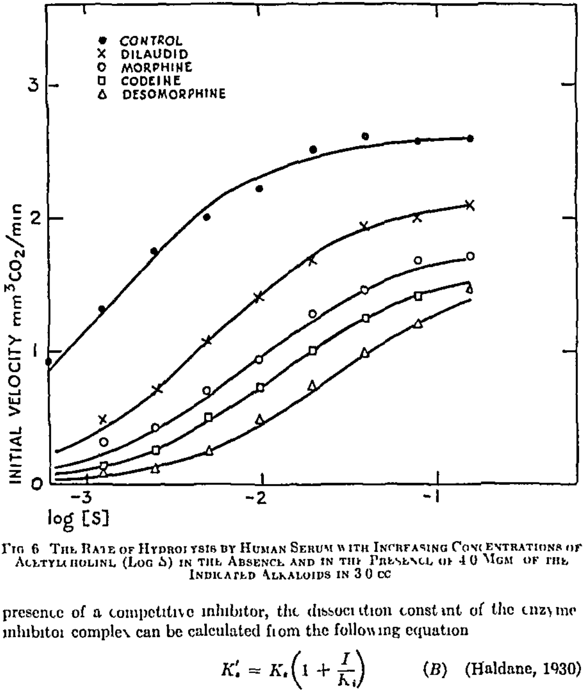

Fig 6 The Rate of Hydrolysis by Human Serum with Increasing Concentrations of Acetylcholine (Log S) in the Absence and in the Presence of 4 0 Mgm of the Indicated Alkaloids in 3 0 cc

presence of a competitive inhibitor, the dissociation constant of the enzyme inhibitor complex can be calculated from the following equation

$$K'_s = K_s\left(1 + \frac{I}{K_i}\right) \qquad (B) \quad \text{(Haldane, 1930)}$$

where I is the molar concentration of inhibitor, K'_s the apparent value for K_s in the presence of I, and K_i the dissociation constant of the inhibitor enzyme complex *K_i for dilaudid was found to be 0012 for morphine 00081, for* codeine .00042, for desomorphine .00018. The relative affinities of the drugs for the enzyme were, consequently, 800, 1200, 2400, 6000 respectively, as compared with the relative affinity of the enzyme for the substrate of 800.

Since our results with human serum did not agree with those of Eadie (6), as regards the presence of non-competitive inhibition and also the value for K_s, we made a series of determinations using 0.04 cc. of dog serum. The results obtained with and without morphine are given in table 2 and figure 7. The data were treated in every respect the same as described for human serum. K_s was found to be .00026, V 2.0, K'_s and V' were found to be .0063 and 1.34. K_i for morphine and dog serum cholinesterase was calculated from (B) and found

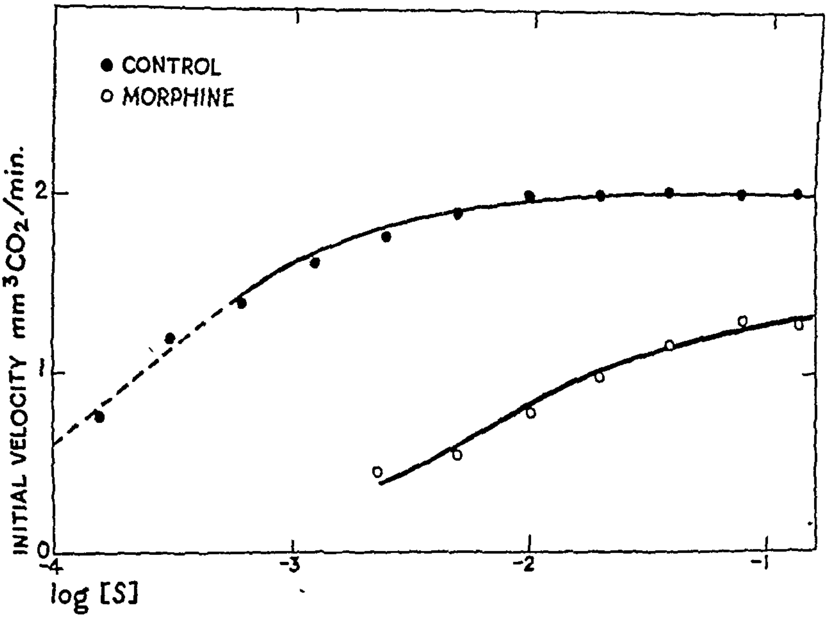

FIG. 7. THE RATE OF HYDROLYSIS BY DOG SERUM WITH INCREASING CONCENTRATIONS OF ACETYLCHOLINE (LOG S) IN THE ABSENCE AND IN THE PRESENCE OF 4.0 MGM. OF MORPHINE IN 3.0 CC.

to have a value of .00015. Thus the enzyme in dog serum appears to have approximately five times as great an affinity for acetylcholine, and also for morphine, as does the enzyme in human serum.

The affinity of the enzyme in dog serum is so great that the reaction proceeds very rapidly even in the presence of very low concentrations of acetylcholine. Thus, as is shown in figure 7, the maximal velocity is realized down to approximately .01M substrate, and falls very slowly at concentrations below that range. Below .001M the yield of CO_2 becomes so small that estimations of initial rates are not very reliable. For this reason the data on the rates at the two lowest concentrations (figure 7) were not used to determine the curve drawn through the points and the curve is essentially an extrapolation as indicated.

DISCUSSION. An interesting feature of the results shown in figures 1–5 is the differences found, both in the degree of effectiveness and the order of effectiveness of the several drugs. For example, in the two tissues from the human subject as shown in figures 3 and 4, there is a definite difference in the degree of inhibition by the alkaloids, leading to a shift in their order of activity. There is also a marked difference in the affinity of dog and human serum cholinesterase for acetylcholine. This indicates that there is more than one type of cholinesterase or that the same enzyme acts differently in different environments. Alles and Hawes (11) described different properties of the enzyme when taken from blood cells or plasma, and Glick (12) has also found differences. These findings offer another possible explanation, besides solubility and permeability, for the variable sensitivity of physiological systems to the inhibitors of cholinesterase. They also emphasize the difficulty involved in drawing conclusions regarding cholinergic activity from experiments on the enzyme from a single source such as blood serum.

In a personal communication, Dr. Eadie has informed us that his published values (6) for K_s and K_i are too high and that more recent experiments have yielded values of .004 and .0006 respectively. These values are still much higher than we found for dog serum but it is possible that there is a wide variation in the properties of cholinesterase in the sera of different dogs. There seems to be little question that the inhibition by the alkaloids studied is in part non-competitive. In fact, we were unable to derive curves to fit the data, even approximately, if we assumed no change in V in the presence of the alkaloids.

SUMMARY

Morphine, codeine, dilaudid and desomorphine inhibit the activity of cholinesterase from the following sources: human, dog or rabbit serum, and human or rabbit brain. The degree of inhibition varies with the source of the enzyme, which might account for the selective action of these drugs on physiological systems. The inhibition is in part competitive and in part non-competitive, judging by the relative reaction velocity at different substrate concentrations. The affinity of the alkaloids for the enzyme in human serum is as great (dilaudid) or greater (codeine, morphine and desomorphine) than the affinity of the enzyme for acetylcholine.

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APPENDIX

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Since the curve,

$$v = \frac{V(S)}{K_s + (S)} \tag{1}$$

is not linear in its parameters, V and K_s, it cannot be fitted directly by least squares. Two alternative forms of this equation (1 and references) which are linear in V and K_s may be written as

$$\frac{(S)}{v} = \frac{K_s}{V} + \frac{1}{V}(S) \tag{2}$$

$$\frac{1}{v} = \frac{1}{V} + \frac{K_s}{V}\,\frac{1}{(S)} \tag{3}$$

In (*2*), $\frac{(S)}{v}$ and (S) are linearly related; in (*3*), $\frac{1}{v}$ and $\frac{1}{(S)}$ are linearly related. Any one of these three equations may be used to estimate the values of K_s and V but, in general, the results will not agree unless the observations are correctly weighted, although frequently the results may not differ appreciably. It should be remembered, however, that in fitting the curves (*2*) and (*3*) by least squares the observations are given a different weight than when equation (*1*) is fitted so that if exactly comparable results are to be obtained the original observations must be weighted if estimates of the parameters are determined from equations (*2*) and (*3*).

Equation (*1*) can be fitted directly by least squares in the following manner:

1. Obtain estimates of K_s and V which will be designated as ${}_oK_s$ and V_o. *These may be taken from the results of other experiments or estimated from* the data by means of equations (*2*) or (*3*).

2. Write the true but unknown values of K_s and V as

$$K_s = {}_oK_s + \Delta K_s \tag{4}$$

$$V = V_o + \Delta V \tag{5}$$

where ${}_oK_s$ and V_o are the estimated values of K_s and V and ΔK_s and ΔV are the respective differences between the true and estimated values. It is necessary to determine ΔK_s and ΔV.

3. The corrections, ΔK_s and ΔV, may be determined from the following equations:

$$\sum \left(\frac{-v_o}{{}_oK_s + (S)}\right)^2 \Delta K_s - \sum \frac{v_o^2}{V_o({}_oK_s + (S))}\,\Delta V = \sum \frac{-v_o(v - v_o)}{{}_oK_s + (S)} \tag{6}$$

$$-\sum \frac{v_o^2}{V_o({}_oK_s + (S))}\,\Delta K_s + \sum \left(\frac{v_o}{V_o}\right)^2 \Delta V = \sum \frac{v_o(v - v_o)}{V_o} \tag{7}$$

where $v_o = \frac{V_o(S)}{{}_oK_s + (S)}$, that is, values of v obtained by substituting the estimated values of K_s and V in equation (*1*). In equations (*6*) and (*7*), v is an observed value.

4. The values of ΔK_s and ΔV calculated from equations (*6*) and (*7*) are substituted in equations (*4*) and (*5*) to obtain estimates of K_s and V. If ΔK_s and ΔV are small compared with ${}_oK_s$ and V_o no further computation is necessary. However, if the corrections are relatively large, ${}_oK_s + \Delta K_s$ and $V_o + \Delta V_o$ may be used as the estimated values in step No. 1 and the entire process repeated.

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THE REDUCTION OF *2,4,6*-TRINITROTOLUENE BY ANIMAL TISSUE IN VITRO

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The occurrence in the urine of reduced products following the administration of symmetrical trinitrotoluene has been known for some time (1, 2, 3, 4). Presumably such reduction should take place in the tissues, although previous workers (3, 5, 12) with the use of bacteria, muscle or spermatozoa were unable to demonstrate anything more than a doubtful reduction which they considered negative. This was attributed to the size of the molecule since[1] the simpler compound nitrobenzene was easily reduced to the hydroxylamine. Work in this laboratory (6) showed that reduced material occurred generally in tissues of the living rabbit following administration of trinitrotoluene (T N T). Whether each tissue had some reducing action or whether one such as the muscle had this function with the blood or lymph distributing the substance throughout the body was not known.

It is therefore of some interest that under suitable conditions the reduction can be shown *in vitro* using various tissues. This paper records such findings. As noted below the partial reduction product *2,6*-dinitro-*p*-toluidine (*2,6*-dinitro-*4*-amino-toluene) has been used as a standard to follow the reduction process. The use of this compound is on a purely empirical basis. In most cases it seemed to behave much like the material formed with respect to time for color development and color quality. Only in the case of the extract of the liver of the cat was a material actually isolated with melting point corresponding to that of the *2,6*-dinitro-*4*-amino compound. The yield was barely 5% of the total diazotizable chromogen. The problem as to what may be formed presents many difficulties even without considering oxidation of the methyl group to the carboxyl group, formation of amino phenols or conjugation with glucuronic acid, as has been suggested (1) for the material excreted in the urine. Some of the simpler possibilities are reviewed in figure 1. Compounds II and VI have been isolated from the urine of rabbits[2] (1, 4) and a strong probability established in favor of a portion of the material so excreted being compound XIII. There was no evidence for the presence of the former in the experiments reported here and the relative stability and insolubility of the azoxy derivative would seem to exclude it. From the present state of knowledge of the course of such reactions as ordinarily carried out in the laboratory the hydroxylamino compound would be expected to be formed as an intermediate but should pass rapidly over to Compound V (or XIII) of the chart

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[1] The test chiefly relied on for detection of reduction by the former workers (5, 12) was the qualitative color reaction in alkaline solution given by many aromatic hydroxylamines. It is decidedly less sensitive than the diazotization procedure used in the present work and is negative with amines.

[2] It should be noted that these compounds were *isolated from* the urine of rabbits. The bulk of the weight of evidence is against their existence as such in the urine and seems to indicate that they may be formed by the extraction process

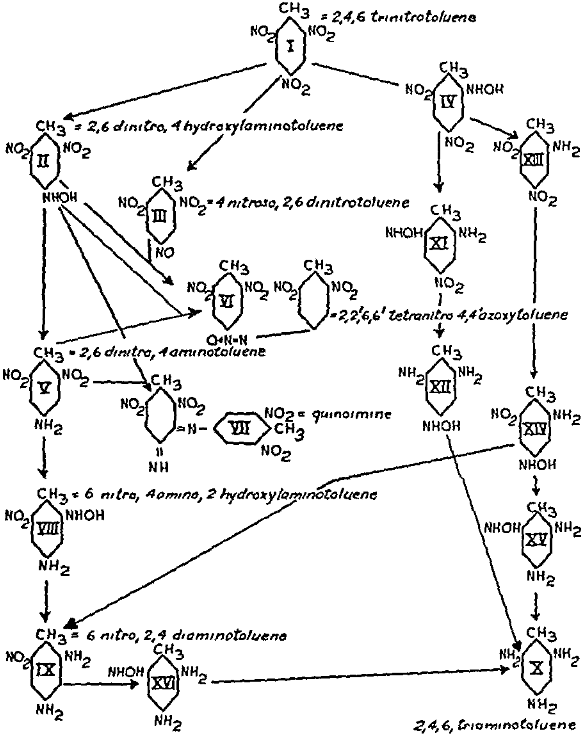

I = 2,4,6 trinitrotoluene
II = 2,6 dinitro, 4 hydroxylaminotoluene
III = 4 nitroso, 2,6 dinitrotoluene
IV
XIII
XI
VI = 2,2',6,6' tetranitro 4,4' azoxytoluene
V = 2,6 dinitro, 4 aminotoluene
VII = quinoimine
XII
XIV
VIII = 6 nitro, 4 amino, 2 hydroxylaminotoluene
XV
IX = 6 nitro, 2,4 diaminotoluene
XVI
X
2,4,6, triaminotoluene

Fig 1

Methods and Materials The animals were used after food had been withdrawn 24 hours They were killed by decapitation and the tissue quickly removed and ground to a smooth paste with the help of sand In the few instances where it was not possible to use the tissue immediately it was kept frozen until used, as this seemed to preserve the activity for a week or more. Two cubic centimeters of a Ringer-phosphate solution (7) per gram of tissue were then added gradually and the grinding continued. For the smaller tissues it was necessary to add four cc. per gram in order to have sufficient extract with which to work. The solution was centrifuged at such a speed as to bring down the major portion of

TABLE 1

Reduction of symmetrical trinitrotoluene by incubation with tissue extracts in vitro at 37.5°C. and pH 7.4 for 1 hour

In expressing results it has been assumed that 1 gram of tissue is the equivalent of 1 cc. in volume, hence that 1 cc. of the suspension represents 0.33 gram of tissue. The figures represent mgm. per 100 grams tissue where 2,6-dinitro-p-toluidine was used as the standard.

TISSUE	ANIMAL USED			
	Rat	Chicken	Dog	Cat
Liver	41	48	40	46
Kidney	22	36	42	29
Heart	26			20
Spleen	10	3		12
Muscle (gluteal)	5	2		2
Ovaries	3			
Brain	2			
Lung	8			
Feces	4			

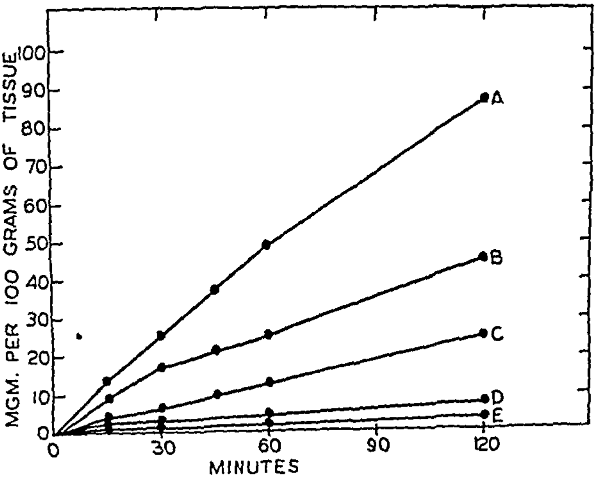


FIG. 2. REDUCTION OF 2,4,6-TRINITROTOLUENE BY RAT LIVER PREPARATION

the cellular material and the supernatant extract used for the experiments detailed below. Unless otherwise specified, 2 cc. of the extract plus 3 cc. of the buffered T N T solution were used for an individual estimation. This made the solution approximately half saturated with respect to T N T in all cases, since regardless of the amount of extract taken the volume was made to 5 cc. or this volume relationship maintained.

A saturated solution of *2 4 6 trinitrotoluene* m p 81 82°C, was prepared by adding 1 gram of the crystalline material to a liter of water and boiling gently for an hour under the

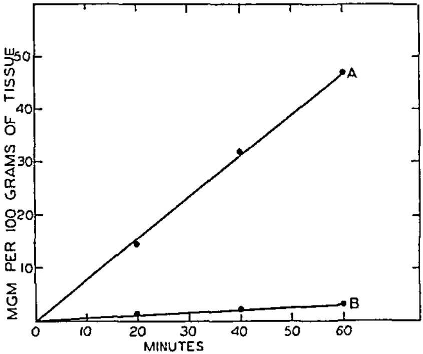


Fig 3 Reducing Action as Affected by Heating to 80°C for 10 Minutes
Curve B is that for the heated preparation

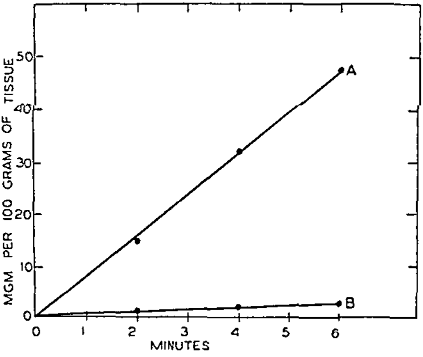


Fig 4 Reducing Action as Influenced by Oxygenation
The portion of the rat liver preparation represented by curve A was under anaerobic conditions

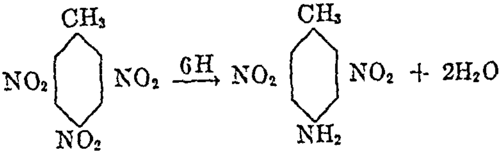
hood After cooling to 37 5°C it was filtered This solution contained 23 mgm T N T per 100 grams of solution and remained almost colorless when stored in the dark for a month The Ringer and phosphate buffer stock solutions had been made ten times the needed concentrations, so that to prepare the buffered T N T solution referred to above for experimental use 10 cc. of the saline were pipetted into 80 of the T N T and 10 of the phosphate buffer added.

Incubation of the tissue was carried out in Thunberg tubes alternately filled 3 times with oxygen-free nitrogen and evacuated. At the end of the experimental period the reaction was stopped and the protein removed by adding twice the volume of 20 per cent trichloracetic acid and an equal volume of water. The contents were shaken immediately and filtered. Five cc. of the filtrate were pipetted into a 25 cc. Erlenmeyer flask, 0.5 cc. 4 N HCl added and diazotization carried out by the method of Bratton and Marshall (8) using half the volumes recommended since one-half the filtrate volume was used. It was necessary to reduce the time of exposure to the nitrite and sulfamate to 0.5 minute each due to the instability of the diazo compound formed (Cf 9, 13). Control incubation with (a) the saline and buffer + T N T (b) extract + saline and buffer gave filtrates containing no diazotizable material, thus showing that the extract was needed for the reduction.

Color comparison was made with standards prepared from 2,6-dinitro-*p*-toluidine (2, 10) using a stock solution of 5 mgm. in 100 cc. of 3% hydrochloric acid. This was stable for a month when kept in the dark. The maximum quantity used was 50 micrograms in 5 cc. and in diluting the stock for an individual standard 2.5 cc. of the 20% trichloracetic acid were added to each to give the amount as in the filtrates. Due apparently to the colloidal nature of the dye the solution does not follow the Beer-Lambert law at higher concentrations. This made it necessary to work at levels from 50 micrograms in 5 cc. downward. The color match with the material formed by the tissues from the T N T was only moderately satisfactory, especially when kidney or liver was the source of the reducing agent. The filtrates from these preparations had more of an orange tint compared to the clear purple-pink of the standard, possibly due to polymerization or quinone formation (11). Color comparison was made in the ordinary Klett colorimeter.

RESULTS. Table 1 contains some data on the reduction of T N T by various tissues of different animals. It was also found that freshly voided normal rat's feces when incubated as above shared this property, thus adding a possible complication to studies in animal feeding experiments.[3]

That the reduction is progressive with time and is a function of the amount of extract used is shown in fig. 2. For simplicity and comparison with the graphs of the other figures curves B, C, D and E of this figure are plotted as though the standard amount of tissue were used. Actually B had half the quantity of A, C, one-fourth, D one-eighth and E one-sixteenth. That the action is almost abolished by heating to 80°C for 10 minutes is shown in fig. 3. Since the reduction should be favored by hydrogen it was suspected that oxygen would depress the reduction or inhibit it. Fig. 4 includes data on this point supporting this postulate. The activity seems optimum under the pH used, but the activity is continued over a considerable range in phosphate buffers as



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[3] Ordinary baker's yeast used as a suspension in phosphate buffer likewise was effective.

shown with the kidney preparation of fig 5 At the upper pH there is browning of the solution due to the alkalinity during incubation but little reduction seemed

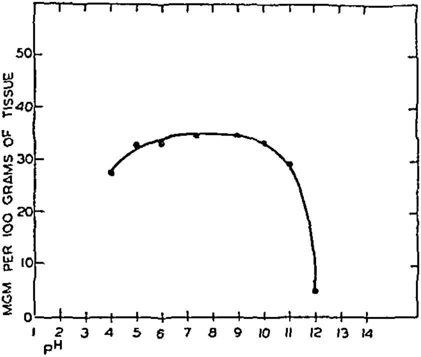

Fig 5 Effect of Various Hydrogen Ion Concentrations on the Reduction by the Rat Kidney Preparation

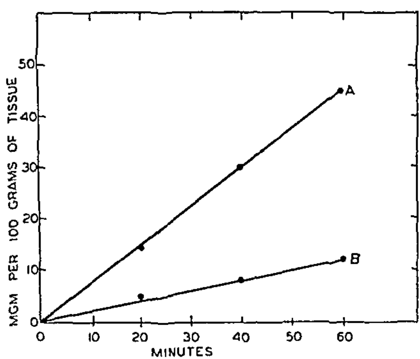

Fig 6 Effect of Washing on the Reducing Activity of a Rat Liver Preparation
Curve A represents the untreated tissue

to take place or if it did the compounds formed were removed by the trichlor acetic acid precipitation of the protein

Much of the activity was lost by washing the partially ground tissue as shown in fig. 6. This was not unexpected since the older literature (3) *records* the ineffectiveness of washed muscle in reducing dinitrobenzene, whereas the untreated muscle in bicarbonate was effective. It was thought that the decrease recorded by curve B might be due to the diffusion out of some dialyzable hydrogen donater, since a source of active hydrogen should theoretically be needed, as mentioned above. Succinate, added as the sodium salt to the washed tissue, brought the activity back to about 80% of the original. The effect of succinate is further borne out by curves M_1 & M_2 of fig. 7. These show the reduction by muscle extract before (M_1) and after (M_2) adding succinate. That much of the reduction might be attributed to some portion of the metabolic system using

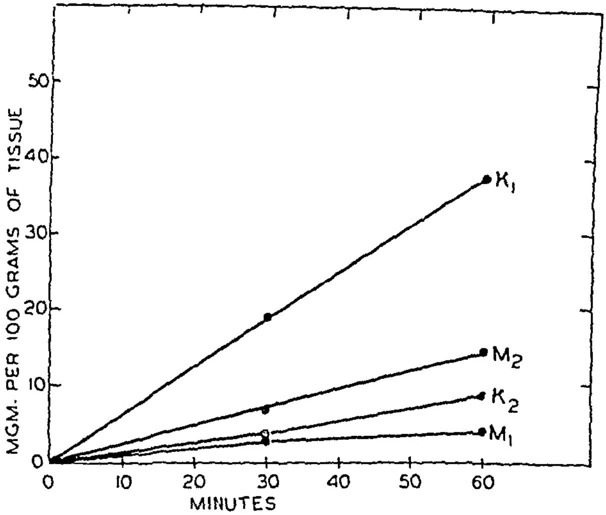

FIG. 7. (a) Effect of added succinate on the reducing activity of a muscle preparation. M_2 contains the added metabolite. (b) Effect of malonate in decreasing reducing activity of a kidney preparation is shown by curve K 2 compared to the untreated preparation of K 1.

succinate would appear from the experiment on the kidney included in fig. 7. Succinate added to the buffer mixture plus T N T without the protein containing extract gave no effect. Added to the extract heated as above likewise gave only a very slight effect. Malonate was found to inhibit the reduction markedly. This ion is generally considered to be a rather specific inhibitor of succinic dehydrogenase. It was accordingly decided to turn to a partially purified enzyme in the hope that this simplification might help with the study of the factors involved. Such a study was undertaken and will be reported separately.

SUMMARY

1. It has been shown that extracts of animal tissues have the power of reducing symmetrical trinitrotoluene *in vitro*. At least a portion of the resulting product or products behaved as a diazotizable amino compound.

2. This reducing power was very largely destroyed by heating to 80°C for 10 minutes It was augmented by anaerobic conditions and very largely inhibited by oxygenation

3 The reducing effect was partially removed by washing and restored by added succinate and discharged again by malonate

4. These facts were interpreted as indicative of the presence of an enzyme concerned in the reduction

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THE CONJUGATION *IN VITRO* OF PHENOL BY GUINEA PIG LIVER

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The demonstration by Richter (1) of a sulfate ester of adrenaline in human urine after oral administration of the hormone indicates that such esterification may normally occur. It may be catalyzed by an enzyme system, sulfosynthase, present in liver, which also esterifies phenol. Recently, Torda (2) studied the hydrolysis of the sulfuric acid ester of phenol by an enzyme extracted from cat muscle. She found that 5% cocaine inhibited the hydrolysis and assumed that it would also inhibit the synthesis. If this were true then the potentiation of adrenaline effects by cocaine might be explained on the basis of this inhibition, provided that esterification of adrenaline by sulfuric acid is the principle mechanism for its inactivation in the body. Torda's results are not conclusive because she studied the hydrolytic instead of the synthetic process, and used a very inactive enzyme preparation and an extraordinarily high concentration of cocaine. Arnolt and de Meio (3) have shown that phenol is esterified by rat liver slices *in vitro* and we therefore studied this esterification in detail and the effect of cocaine on it. It was necessary to use phenol instead of adrenaline because of the instability of the latter in solution.

Experimental. Guinea pig liver slices were used in most of the experiments. They were suspended in a Ringer solution containing 100 parts 0.9% NaCl, 4 parts 1.15% KCl, 3 parts 1.12% $CaCl_2$, 1 part 2.11% $KHPO_4$, 1 part 3.82% $MgSO_4 \cdot 7H_2O$ and 21 parts 1.3% $NaHCO_3$. The solution was saturated with a 95% oxygen 5% carbon dioxide mixture and the experiments were carried out in this atmosphere with the slices suspended in 4.0 cc. of solution in 50 cc. Erlenmeyer flasks and shaken at 37°. At the end of the experiment 1.0 cc. 20% trichloracetic acid was added, the slices removed and the solution centrifuged and then poured off from the precipitate. The volume was made up to 10 cc. with distilled water and 0.5 cc. saturated NaCl and the phenol remaining estimated according to the method of Theiss and Benedict (4). To another flask before adding the water 0.25 cc. concentrated HCl was added and the tube placed in a boiling water bath for 10 minutes to hydrolyze any esterified phenol. The HCl was then neutralized by an appropriate amount of NaOH and the phenol estimated. The difference between the values obtained with and without boiling represented the amount of esterification. The weights of the slices in each flask checked to within 10%. As the slices themselves without added phenol gave a light yellow color with the reagent it was necessary to compensate for this by shaking slices without phenol under identical conditions and adding appropriate amounts of phenol just before the trichloracetic acid. These were used as standards and the experimental tubes were read against the corresponding standard so that there was never more than a 15% difference in the color of the two solutions. This eliminated the error due to the light yellow color. This color was not affected by boiling the solution before the addition of the reagents or by the length of time the liver slices were shaken.

Phenol disappears when added to liver slices under the conditions outlined above. This disappearance might be caused by adsorption of the phenol onto proteins, by oxidation, or by conjugation As shown in table 1 between 80–90% of the added phenol is recovered when it is shaken with broken cell suspensions under identical conditions Since such suspensions offer a much greater protein surface than slices, this experiment indicates that phenol is not adsorbed onto protein under these conditions If the phenol is shaken with slices anaerobically with nitrogen substituted for oxygen 80–90% can be recovered This also indicates that no appreciable adsorption has occurred and that oxygen is necessary for the disappearance As shown in table 2, 17–38% of the phenol that disappears is conjugated The rest is probably oxidized because phenol consistently increases the oxygen uptake of liver slices but to a very small extent The possibility that it may disappear in other ways has not been ruled out

Since phenol may be changed by the cell in at least two different ways, oxidation or conjugation, the effect of the weight of slices used in each vessel was studied The results are shown in table 2 As the weight of slice is decreased the percentage conjugation is increased This may be interpreted as meaning that the enzyme system responsible for the oxidation is present in the cells in a lower concentration than the enzyme responsible for the conjugation and thus as the number of cells is decreased the oxidation becomes the limiting factor If the phenol concentration is too large relative to the amount of tissue present, the percentage disappearance and conjugation becomes less, indicating that the higher concentrations are toxic The best results in general were obtained with about 160–180 mgm wet weight of slices and 0 2 mgm phenol in 4 0 cc

TABLE 1

The effect of various conditions on the conjugation of phenol by guinea pig liver

The vessels were shaken for 90 minutes at 37°

WEIGHT	PHENOL ADDED	PHENOL RECOVERED	PHENOL RECOVERED AFTER HYDROLYSIS	PHENOL CONJUGATED	PHENOL CONJUGATED / PHENOL DISAPPEARED
mgm	*mgm*	*mgm*	*mgm*	*mgm*	%
215 slices	0 2	0 035			
204	0 2		0 083	0 048	29
214 slices	0 2	0 173			
217 boiled 5 min	0 2		0 170	0 000	0
210 slices	0 2	0 166			
215 anaerobic	0 2		0 166	0 000	0
220 broken cell	0 2	0 176			
220 suspension	0 2		0 177	0 000	0

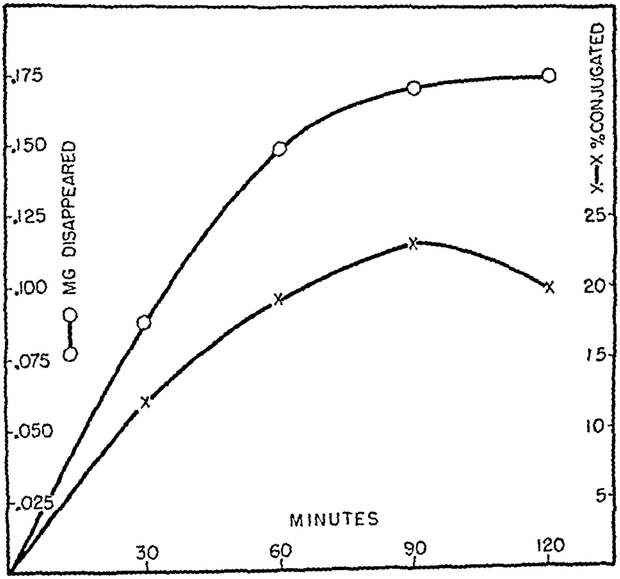
Figure 1 shows the curve of the disappearance and conjugation as related to time The disappearance proceeds in a way consistent with an enzymic reaction The conjugation process rises to a maximum at about 90 minutes and then falls off This is a regular phenomenon and can be explained if it is assumed that hydrolysis of the conjugated phenol is proceeding at the same time as synthesis occurs. At the beginning of the experiment when the maximum amount of free phenol is present synthesis will proceed at a rate determined only by the

TABLE 2

The effect of the weight of tissue slices on the percentage conjugation of phenol by guinea pig liver

The vessels were shaken 90 minutes at 37°.

WEIGHT	PHENOL ADDED	PHENOL RECOVERED	PHENOL RECOVERED AFTER HYDROLYSIS	PHENOL CONJUGATED	PHENOL CONJUGATED / PHENOL DISAPPEARED
mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	%
84	0.2	0.077			
80	0.2		0.124	0.047	38
157	0.2	0.041			
160	0.2		0.075	0.031	21
221	0.2	0.024			
224	0.2		0.054	0.030	17



IG. 1. The Time Curves of the Disappearance and Percentage Conjugation of 0.2 MGM. Phenol in the Presence of 160 MGM. Wet Weight of Guinea Pig Liver Slices at 37°

activity of the enzyme. But in the course of time as the free phenol concentration decreases because of oxidation and conjugation the equilibrium will be shifted and some of the esterfied phenol may then be hydrolyzed and the phenol thus set free either resynthesized or oxidized. As oxidation or some other process accounts for most of the disappearance of the phenol, no evidence of synthesis would be expected if the experiment was allowed to proceed long enough.

Phenol can be esterified either as a sulfate or glycuronate.[1] Slices were therefore suspended in Ringer solution containing all the normal ingredients except $MgSO_4$. $MgCl_2$ was substituted in an equivalent concentration. The results are shown in table 3. In the absence of sulfate little or no synthesis takes place. This experiment indicates that the cell is able to use preformed sulfate and that a sufficient amount is available despite the presence of calcium. Increasing the sulfate concentration does not increase the percentage conjugation. Cystine and methionine added in the absence of sulfate did not cause synthesis indicating that they do not act as a source of sulfate under these conditions.

TABLE 3

The effect of sulfate on the conjugation of phenol by guinea pig liver slices

The experiment is given in triplicate. The vessels were shaken for 90 minutes at 37°.

WEIGHT	PHENOL ADDED	PHENOL RECOVERED	PHENOL RECOVERED AFTER HYDROLYSIS	PHENOL CONJUGATED	PHENOL CONJUGATED / PHENOL DISAPPEARED	
mgm	*mgm*	*mgm*	*mgm*	*mgm*	%	
181	0.2	0.041				Sulfate added
192	0.2		0.082	0.041	26	
188	0.2	0.040				
188	0.2		0.086	0.046	29	
188	0.2	0.050				
178	0.2		0.090	0.040	27	
189	0.2	0.072				No sulfate added
191	0.2		0.072	0.000	0	
182	0.2	0.073				
182	0.2		0.077	0.004	3	
190	0.2	0.063				
189	0.2		0.077	0.014	10	

The effect of cocaine on the conjugation was then tried. It was, of course, impossible because of the osmotic effects, to use 5% solutions. A concentration of 1.0 mgm per cc. was found possible which is equivalent to M/300 cocaine HCl. The results are shown in table 4. Cocaine definitely inhibits the disappearance of phenol but has no effect on the percentage conjugated. Since the oxidation is inhibited it cannot be argued that the failure of cocaine to inhibit esterification is due to its inability to penetrate into the cells.

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[1] Lipschitz and Bueding (5) showed that phenol was conjugated to some extent with glycuronic acid by liver slices in the absence of sulfate. This conjugation was small compared to that of borneol.

In the guinea pig the intestine and the kidney fail to cause any appreciable disappearance or conjugation of phenol. However, in the rat, Arnolt and de Meio found that the intestine conjugated phenol better than liver, and that the kidney had a small effect. They were unable to show that any conjugation took place in muscle.

Discussion. Since these experiments were done with phenol instead of adrenaline the results apply to adrenaline only by inference. However, it is reasonable to assume that the esterification of both compounds with sulfuric acid is brought about by the same enzyme. The synthetic activity of this enzyme has been demonstrated in the intestine and liver but in no other organ. The hydrolytic activity of the enzyme, or rather the hydrolysis of phenol sulfate, has been demonstrated in a number of organs including muscle. But this hydrolysis is extraordinarily slow, 2–5% of the added substrate being hydrolyzed in 3–5 days at 37°. The present evidence, concerning the distribution and activity of the enzyme therefore does not warrant the conclusion that it is responsible for what must be the rapid inactivation of adrenaline in the body. Our results with cocaine, which were obtained under as nearly as possible physiological conditions, do not explain the potentiation by this drug of adrenaline effects if the esterification of adrenaline by sulfuric acid is the principle method of its inactivation. In fact it is more reasonable to assume that ingested adrenaline arriving at the liver *via* the portal circulation is conjugated as phenol is and that this conjugation is a special case and does not account for the normal inactivation of the hormone in the body. Since this paper was written, Torda (6) has studied the effect of cocaine on the excretion of phenol in the cat. Cocaine has no effect on the elimination or conjugation of phenol produced endogenously. Injection

TABLE 4

The effect of M/300 cocaine HCl on the disappearance and conjugation of phenol by guinea pig liver slices

The vessels were shaken 90 minutes at 37°. The experiment is given in duplicate.

WEIGHT	PHENOL ADDED	PHENOL RECOVERED	PHENOL RECOVERED AFTER HYDROLYSIS	PHENOL CONJUGATED	PHENOL CONJUGATED / PHENOL DISAPPEARED	
mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	%	
154	0.2	0.068				Controls
170	0.2		0.098	0.030	23	
156	0.2	0.072				
166	0.2		0.097	0.025	22	
166	0.2	0.100				Cocaine
158	0.2		0.124	0.024	24	
169	0.2	0.100				
154	0.2		0.119	0.019	19	

of phenol into the normal animal causes 41% more free phenol to be excreted in the urine in the first 48 hours In the presence of cocaine only 6% more free phenol is excreted The fact that injected phenol does not appear in the urine in significant amounts in cocainized cats raises the question of its fate but does not prove that it has not been conjugated, particularly as cocaine does not affect the conjugation of endogenous phenol.

SUMMARY

1. Phenol disappears when added to guinea pig liver slices in Ringer bicarbonate solution shaken at 37° in 95% oxygen 5% carbon dioxide. 20–30% of the amount that disappears is conjugated.

2. Thermolabile enzyme systems are responsible for the disappearance and conjugation. Neither process occurs anaerobically. The effects of the amount of tissue and substrate concentration are described.

3. In the absence of added sulfate no conjugation occurs Cystine or methionine cannot act as sources of sulfate under the experimental conditions

4 M/300 cocaine HCl inhibits the disappearance of phenol but has no effect on the percentage conjugated.

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EFFECT OF ANESTHESIA ON LYMPH FLOW (LOCAL PROCAINE, ETHER, PENTOBARBITAL SODIUM)

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Evidence that anesthesia causes a shift in the ratio of plasma volume to interstitial fluid volume can be found in a number of studies (1, 2, 5, 6, 7, 8, 9, 11, 14, 16). Moreover, the direction the change appears to take depends upon the nature of the anesthetic agent employed. This suggested to us that an effect of anesthesia on lymph flow might be demonstrated, and on examining this possibility we have observed striking alterations in the rate of lymph flow, depending upon whether local, ether or barbiturate anesthesia is employed. In addition to reasons of pharmacological interest there are practical reasons why such a demonstration is helpful. For example, it appears to provide a useful step in the explanation of why barbiturates delay the onset of shock in comparison with ether, under the circumstances of the experiments of Seeley, Essex and Mann (15), and why a barbiturate significantly curtails the weeping from burned surfaces, as shown by Beecher and McCarrell (3).

METHODS. *Animals.* This study is based upon observations made in 26 mongrel dogs When local anesthesia was used, mongrel hounds were more easily adaptable and more acquiescent than some other breeds to the rhythmic motion of the head necessary for the collection of lymph. The dogs were allowed water but were fasted for 18 hours before the experiment. Immediately preceding the experiment each animal was given physiological saline intravenously to the extent of 20 cc. per kgm. over a period of an hour.

Anesthesia. When the lymph was collected under local anesthesia, the region of the cervical lymphatics was infiltrated with 2.0 cc. of 1.0% procaine hydrochloride. A femoral artery was cannulated under local procaine anesthesia for intermittent determination of mean arterial pressure.

In the cases where the lymph was first collected under local anesthesia (tables 1 and 2), the subsequent induction of ether was carried out by open cone. With the onset of general anesthesia, an intratracheal tube was introduced through the mouth and this was connected with an ether bottle. The data of tables 1 and 2 were obtained with the animals breathing room air.

When the ether was induced at the beginning of the experiment (table 3), this was accomplished in an ether box. Following induction, ether was administered through an intratracheal tube, in a closed system, with to-and-fro breathing through a soda lime cannister. About 95% oxygen was administered when a closed respiratory system was used in the case of both the barbiturate and the ether (tables 3 and 4).

Pentobarbital sodium (4% solution; 20–40 mgm/kg total dose) was injected intravenously as needed to maintain a steady level of anesthesia. About the same oxygen concentration in the inspired air and the same volume of dead space was maintained (intratracheal tube) when the barbiturate was used as in the case of the ether. The dead space was kept at a minimum. Both of the general anesthetic agents were administered in sufficient dose to maintain a moderately light level of surgical anesthesia, where the corneal reflex was present but distinctly depressed.

Lymph collection. The lymph was collected from the cervical lymphatics by the method of McCarrell (12) By means of a motor the head is made to nod through a fixed arc at the rate of about 16 times per minute Leads from the motor were attached to a leather muzzle in the case of the collection under local anesthesia When general anesthesia was employed throughout the experiments the motor leads were attached to holes drilled in the eye teeth Fairly exact reproducibility of motion of the head of a given dog from one period to another is essential for quantitative comparisons of lymph flow from one collecting period to another

Cannulae were introduced into each main cervical lymphatic except as mentioned below In case double lymphatics were present on a side one was tied off A few grains of dry heparin were introduced into each cannula and the lymph collected at 5 to 15 minute intervals placed in stoppered tubes and the weight determined The flow was expressed in milligrams per minute averages were based upon approximately 45 minute collections

In a survey of the experiments in which due to technical mishap lymph was collected from only one cervical lymphatic (the non cannulated lymphatic being tied off) the average flow was found to be 62% (8 dogs) of that obtained when both cervicals (18 dogs) were cannulated With this evidence it seemed reasonable to add one third to the observed lymph values collected from a single vessel in order to compare these data with those obtained when both cervicals were cannulated successfully care was taken to have the same number of such experiments in both control and test groups under comparison There were only a few such experiments they are clearly marked in the tables

Ether induction by cone was always followed by a greatly increased lymph flow due in part to the activity of the animal during induction In determining the average flow during ether anesthesia collections made during the first 30 minutes following the induction of the anesthetic were discarded In order to make fair comparisons with the flow under the barbiturate a similar delay was made in the barbiturate experiments

Lymph was collected in four series of animals (a) local anesthesia followed by barbiturate and then ether anesthesia (table 1) (b) local anesthesia followed by ether and then barbiturate anesthesia (table 2) (c) ether alone and barbiturate anesthesia alone (table 3) and finally (d) barbiturate followed by ether anesthesia (table 4) The data of tables 3 and 4 are now supplementary although they were obtained first before we had learned that it was possible to make satisfactory observations under local anesthesia

The mean arterial blood pressure was measured in the femoral artery at about half hourly intervals Consecutive experiments are represented here except in the infrequent case where the mean arterial blood pressure failed to remain normal and relatively constant or where under local anesthesia the animals failed to lie in a quiet acquiescent state The protein content of the lymph and the plasma (arterial blood) were determined refractometrically The hematocrit was also determined in arterial blood Rate and depth of respiration were carefully observed

RESULTS The results are presented in tables 1 through 4[1] Data in tables 1 and 2 show that the reduction in lymph flow during pentobarbital anesthesia is accompanied by decreased hematocrit and plasma protein values The increased lymph flow during ether anesthesia is accompanied by increased hematocrit and plasma protein values Since these changes are so preponderately in the same direction and are consistently reversible by a change in anesthetic they must be regarded as significant While the hematocrit changes might be construed to mean various things the changes in plasma protein concentrations suggest that *under the barbiturates fluid moves from the tissues into the blood stream with the*

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[1] The data of tables 1 and 2 were obtained in 1943 those of tables 3 and 4 were obtained two years previously Although the relationships are in the same direction as referred to below it is evident that lymph protein concentrations vary rather widely with different lots of dogs and probably with season as well

TABLE 1

Local anesthesia followed by barbiturate anesthesia and then ether

NO.	WEIGHT	SEX	PROCAINE HCl						PENTOBARBITAL SODIUM							ETHER					
			Lymph		Blood				Lymph		Blood					Lymph		Blood			
			Flow	Protein	Hematocrit	Protein	Mean arterial pressure	Respiration/min.	Flow	Protein	Hematocrit	Protein	Mean arterial pressure	Respiration/min.	Dose	Flow	Protein	Hematocrit	Protein	Mean arterial pressure	Respiration/min.
	kg.		*mgm./minute*	%	%	%	*mm. Hg*		*mgm./minute*	%	%	%	*mm. Hg*		*mgm./kg.*	*mgm./minute*	%	%	%	*mm. Hg*	
1	12.5	♀	127.9	1.81	29.7	5.83	128	16	97.7	1.94	28.6	5.31	140	15	32	274.7	1.63	34.4	6.25	148	24
2	12.5	♀	120.0	2.74	44.3	6.25	136	30	64.7	2.93	36.2	5.97	123	26	31	180.8	2.17	47.5	6.68	136	32
3	12.7	♀	109.2*	2.19	47.4	6.14	141	52	47.1*	2.47	41.7	5.57	133	18	37	190.9*	1.97	53.7	6.23	128	25
4	11.5	♂	150.1	3.16	32.8	6.16	133	24	57.8	3.16	32.4	6.03	137	14	31	187.2	2.66	42.3	6.62	143	26
5	10.5	♂	79.7	2.41	49.0	5.68	121	16	50.9	2.88	46.1	5.60	127	12	27	98.0	2.61	45.0	5.54	133	15
Average.			117.4	2.46	40.6	6.01	132	28	63.6	2.68	37.0	5.70	132	17	32	186.3	2.21	46.3	6.26	138	21

* Flow from one side; ⅓ added to observed values, see text.

TABLE 2

Local anesthesia followed by ether and then barbiturate anesthesia

NO.	WEIGHT	SEX	PROCAINE HCl						ETHER						PENTOBARBITAL SODIUM						
			Lymph		Blood				Lymph		Blood				Lymph		Blood				
			Flow	Protein	Hematocrit	Protein	Mean arterial pressure	Respiration/min.	Flow	Protein	Hematocrit	Protein	Mean arterial pressure	Respiration/min.	Flow	Protein	Hematocrit	Protein	Mean arterial pressure	Respiration/min.	Dose
	kg.		*mgm./minute*	%	%	%	*mm. Hg*		*mgm./minute*	%	%	%	*mm. Hg*		*mgm./minute*	%	%	%	*mm. Hg*		*mgm./kg.*
6	15.5	♀	191.1	2.37	59.7		131	20	380.0	1.96			150	37							
7	6.9	♀	72.8	2.99			130	31	87.7	2.51	45.8	7.13	135	60	17.0	3.16	37.8	6.25	113	93	21
8	12.3	♂	122.3	3.23	41.0	5.88	117	30	123.4	3.03	58.7	6.68	121	54	56.4	3.57	58.0	6.40	111	54	20
9	10.5	♂	102.2	2.17	32.0	6.49	115	12	181.7	2.04	39.8	6.64	117	53	76.9	2.29	39.7	6.77	125	45	23
10	13.0	♀	102.6	4.25	36.8	7.13	106	52	157.8	4.27	50.5	8.06	112	120	29.2	4.64	43.0	7.33	100	112	19
11	9.8	♀	79.5	2.23	31.4	6.31	128	21	85.7	2.04	56.0	7.35	130	72	31.9	2.62	50.5	6.11	117	26	24
Average . . .			111.8	2.87	41.4	6.46	122	28	169.4	2.64	50.0	7.17	127	66	42.3	3.26	47.8	6.57	113	66	21

reverse process occurring under ether. These trends are in agreement with plasma volume changes during anesthesia. See especially references (5) (8) and (16). These hematocrit and protein concentration changes have often been referred to previously. (See the papers mentioned in the introduction.) It is significant that these changes occurred irrespective of the order in which the anesthetics were administered.

TABLE 3

Barbiturate anesthesia alone and ether alone

PENTOBARBITAL SODIUM				ETHER			
No	Average lymph flow	Average lymph protein	Mean arterial press	No	Average lymph flow	Average lymph protein	Mean arterial press
	mgm/min	%	*mm Hg*		*mgm/min*	%	*mm Hg*
12	39.0	3.49	133	17	163.4	1.69	92
13	37.5	3.74	111	18	71.5	3.18	89
14	47.5*	2.60	147	19	92.1	4.19	118
15	58.4*	3.03	120	20	114.1*	2.29	85
16	72.1	2.88	113	21	69.2*	2.88	110
Average	50.9	3.15	125		102.1	2.85	99

* Flow from single lymphatic, ⅓ added, see text

TABLE 4

Barbiturate anesthesia followed by ether

	AVERAGE LYMPH FLOW		AVERAGE LYMPH PROTEIN		MEAN ARTERIAL PRESSURE	
	Pentobarbital sodium	Ether	Pentobarbital sodium	Ether	Pentobarbital Sodium	Ether
	mgm/min	*mgm/min*	%	%	*mm Hg*	*mm Hg*
22	28.7*	45.1*	3.53	3.60	125	160
23	35.9*	54.0*	2.11	2.14	90	74
24	42.7	90.3	4.51	3.67	120	104
25	23.2*	45.9*	4.73	4.45		
26	64.1	114.4	3.26	2.41	124	135
Average	38.2	69.9	3.63	3.25	115	118

* Flow from single lymphatic, ⅓ added, see text

The percentage of total protein was so consistently higher in the lymph collected during barbiturate anesthesia and so consistently lower during ether anesthesia and the protein concentration was so consistently reversed by changing the anesthetic agent that the difference must be considered significant, although probably not great enough to be of importance unless subsequent study should show that the protein composition is altered, as for example if one protein fraction were missing in the ether lymph. As one might have anticipated the protein concentrations in the lymph and the plasma bear an inverse relationship to each other.

The data presented demonstrate that the volume of lymph flow in unit time can be greatly influenced by anesthetic agents: a barbiturate reduces the flow to about one-half the flow under local anesthesia and ether increases it about one-half above the flow under local anesthesia. Before some implications of these data are discussed two questions must be considered. (a) Since the lymph is collected in the cervical region, can variations in respiration under the two general anesthetic agents account for the observed differences in flow? (b) Can differences in muscle tension of the neck muscles account for the results obtained?

(a) Careful observations were made of the rate and depth of respiration. In 4 (no. 1, 2, 4 and 5) of the 11 experiments of tables 1 and 2 the respiratory activity did not show great differences under the three anesthetic agents, while typical changes in lymph flow, depending upon the anesthetic agent, occurred.[2] A further check on a possible respiratory effect was made by separating the local anesthesia data of tables 1 and 2 into two groups according to the respiratory rate. In group 1 the average rate was 18.6 per minute (6 experiments: variation, 12–24) with an average flow of 121.7 ± 18.1 mgm. per minute. In group 2 with an average respiratory rate of 39 (5 experiments: variation, 30–52) the flow was 105.4 ± 9.0 mgm. per minute. The difference in flow under the two circumstances is not significant, although the difference in respiratory rate was great. It was observed, however, that both panting and the forced respiration of excitement were associated with increased lymph flow. These conditions were avoided in these experiments.

(b) If data comparing local with barbiturate anesthesia only had been obtained, it might be argued that changes in muscle tone and tension as a result of the induction of general anesthesia possibly might account for the observed reduction in flow. This cannot be the case since general anesthesia produced by different agents followed the local anesthesia and with opposite effects. Typical effects were observed irrespective of the order one agent followed another.

The observed anesthetic effects on lymph flow cannot be accounted for by respiratory changes or by differences in muscle relaxation.

Discussion. *1.* In the literature describing studies of the lymphatic apparatus frequent reference has been made to "dry" animals, that is, animals which yielded little or no lymph on cannulation of their vessels. In as much as the majority of these investigations were carried out under barbiturate anesthesia, it appears probable, as a result of the present findings, that the barbiturate anesthesia rather than a pecularity of the animal in question was a considerable factor in this situation. Further, it can be suggested that under some circumstances where a profuse flow of lymph is desired, ether anesthesia will promote it.

2. In 1936, Seeley, Essex and Mann (15) reported that surgical shock produced by standardized intestinal trauma occurred much sooner when ether anesthesia was employed than when a barbiturate was used. They called attention to the fact that weeping of the traumatized intestinal surface, as well as the flow of saliva, was much greater under the former than under the latter agent. Beecher, McCarrell and Evans (4) repeated the experiment. They confirmed the observation of Seeley, Essex and Mann and also observed the same conspicuously greater fluid loss from the traumatized intestinal surface when ether was used than when barbiturate anesthesia was employed. The more rapid onset of shock under ether as compared with a barbiturate was possibly due, as Seeley, Essex and Mann suggested, to the greater fluid loss in the one case as compared with the other. The findings in the present experiments strongly support this view. If this is the correct explanation, the shock times measured under circumstances of local anesthesia compared with barbiturate would be less striking than when the comparison is made between ether and a barbiturate, for as already pointed out, the barbiturate diminishes the lymph flow and fluid loss from this important source, whereas ether greatly increases it. The contrast between the barbiturate and the ether fluid loss is far greater than would be the contrast between the fluid loss under barbiturate and local or no anesthesia, if the above view is correct.

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[2] Experiments were carried out in curarized animals where artificial respiration was conducted at widely varying rates without change in lymph flow. It has not seemed worth while to include the details of these experiments here since the intermittent positive pressure type of respiration is so unlike the normal process.

3. The present observations of the effect of a barbiturate in reducing lymph flow provide a likely explanation for the significant reduction in weeping of a burned surface effected by a barbiturate, reported by Beecher and McCarrell (3).

4. While the present demonstration provides a probable basis for one step in the explanation of how barbiturates reduce the fluid loss from traumatized surfaces, the fact that lymph flow is less under a barbiturate than under local anesthesia remains unexplained. The most probable explanation for this appears to be that barbiturates reduce the extent of the blood capillary filtering surface. Some evidence supporting this view has been obtained, but it has not yet been satisfactorily demonstrated.

CONCLUSIONS

1. When lymph is collected under standardized conditions, it is observed that in comparison with the volume flow under local anesthesia that (a) a barbiturate, pentobarbital sodium, reduces this flow about 50% and (b) that ether anesthesia increases the flow about 50% above that found under local anesthesia.

2. The increased lymph flow under ether is associated with increased hematocrit and plasma protein values, the decreased lymph flow under the barbiturate is associated with decreased hematocrit and plasma protein values. These plasma protein changes support the view that under a barbiturate there is a shift of fluid from the tissues to the blood stream, with the reverse process occurring under ether. The concentration of protein in lymph collected under a barbiturate is appreciably higher than it is under ether.

3. It is probable that the often referred to "dry" animals encountered in lymph studies are frequently to be explained by the fact that most of these studies have been conducted under barbiturate anesthesia. Ether can be utilized to produce a profuse lymph flow when this may be desirable.

4. The observations reported here of the effect of a barbiturate and of ether anesthesia on lymph flow appear to provide a useful step in the explanation of

why surgical shock appears sooner under ether than under a barbiturate in the intestinal trauma experiments of Seeley, Essex and Mann.

5. This study provides a likely explanation for the significant reduction in weeping by a burned surface effected by a barbiturate, as previously reported by Beecher and McCarrell.

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